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## FINE NEEDLE CYTOLOGIC BIOPSY IN DISEASES OF THE SALIVARY GLANDS

By

THORBJÖRN BERGE and NELS SÖDERSTRÖM

Received 13 XI 62

In the diagnosis of salivary gland disease biopsy is often desirable, but resorted to only reluctantly because of cosmetic considerations, risks of nerve lesions, salivary fistulas, etc. Such contra indications may be avoided using *fine needle biopsy* for cytologic diagnosis. Infor-

and our own experiences with the method sufficiently encouraging to merit a short report, this all the more as the literature on the subject is still very scanty (Lopes Cardozo 1954, Passeyro 1956, Söderström 1958).

### METHOD

For the puncture we have used ordinary hypodermic needles with an outer dia-  
is were  
tiguous  
tissue  
and  
routine

### MATERIAL

Our clinical material consists of 90 patients with salivary swellings of different type. Most of the patients were treated in the out patient services of the departments of otitis, internal medicine and general surgery of this hospital. The hospital has no specialized tumor clinic and the representation of different types of salivary gland diseases in this material can be assumed to reflect their relative frequencies in general practice with the exception that epidemic parotitis is probably not represented.

### CYTOLOGIC FINDINGS

The main constituent of most punctates is peripheral blood. A gain of clear salivary fluid indicates the presence of cystic cavities. In the absence of tumor or inflammatory reactions the smears are usually very poor in tissue cells. The typical finding in such smears is a small

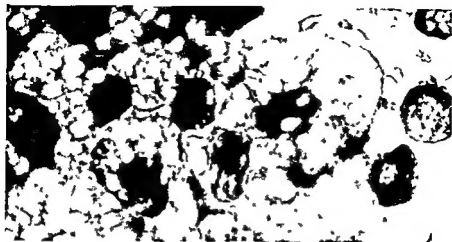


Fig 1

Detail of a cluster of large glandular cells from a salivary gland (submaxillary)  
The cytoplasmic vacuoles somewhat larger than usual ( $\times 1000$ )

number of *large glandular cells* with a densely vacuolated "foamy" cytoplasm, sometimes containing small granules staining dark violet (Fig 1, 2) Such cells often occur in groups, to be recognized as remnants of glandular acini, which may be united with fragments of efferent ducts, built up by *small, basophilic, cuboid cells* At the point of junction between efferent ducts and acini somewhat larger basophilic cells sometimes seem to represent *transitional forms* between duct epithelium and gland cells (Fig 3) When occurring isolated the basophilic cells have a very nonspecific appearance and may be mistaken for lymphocytes

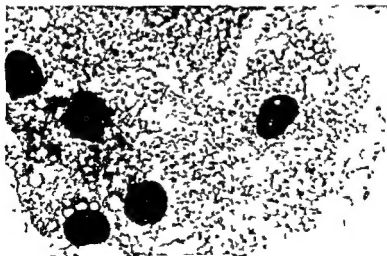


Fig 2

Large glandular cells from the parotid gland Detail of a burst acinus The typical granulation (paravacuolar granulation) is not always as distinct as here ( $\times 800$ )



Fig 3

Cuboid duct cells (left) transitional cell (centre) and large glandular cell (right)  
(Parotid gland punctate  $\times 1000$ )

The gland cells may be extremely scarce but their appearance is sufficiently characteristic to permit the identification of the structure punctured as a salivary gland. This simple identification is often a performance of clinical importance—salivary gland swellings are often mistaken for being lymphomas etc before the puncture.

*Tumor punctates* are usually rich in cells. A very typical picture is offered by *pleomorphic adenomas* which are dominated by a rather uniform population of medium-sized basophilic cells, with dense nuclei, sometimes similar to the transitional cells described above (Fig 4-5), in some cases they may be similar to plasma cells. In punctates from female patients the nuclei often contain a distinct sex chromatin in

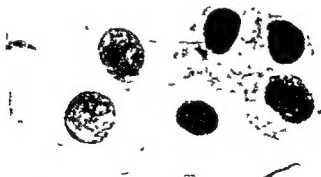


Fig 4

Tumor cells from a case of pleomorphic adenoma ( $\times 1000$ )



Fig 1

Detail of a cluster of large glandular cells from a salivary gland (submaxillary)  
The cytoplasmic vacuoles somewhat larger than usual ( $\times 1000$ )

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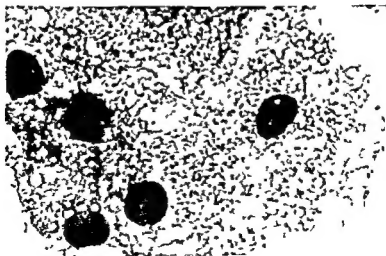


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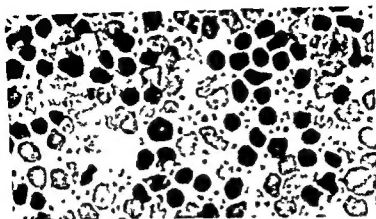


Fig 7

Detail of a parotid gland punctate smear from a case of Mikulicz disease. The smear had the character of a lymph node punctate dominated by lymphocytes, polymorphocytes and lymphoblasts.

In some cases unusually moderate degree of nuclear anisomorphism may indicate an increased tendency to proliferation (and thus to recurrence after surgical removal). There was in our material a good agreement in this respect between the impression from the cytologic picture and the subsequent pathological anatomical statement based on the histology of the tumor.

An overt cancer cytology represented by poorly differentiated and grossly polymorphic cells was present only in a few of our cases. The



Fig 8

Giant cell of the Langhans type from a case of sarcoidosis (parotid gland punctate  $\times 900$ )

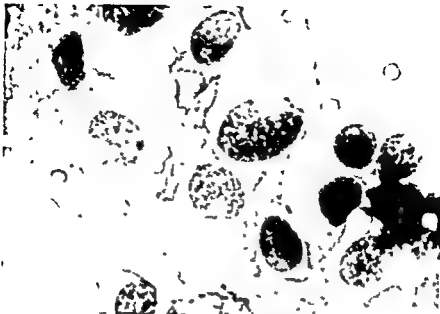


Fig 5

Tumor cells from a case of pleomorphic adenoma. A distinct sex chromatin in 2 of the cells ( $\times 1000$ )

marginal position. The tumor cells usually occur together with mucoid substance staining brightly red, sometimes covering large areas of the smears, sometimes seen only as discrete stellate deposits (Fig 6). The attribute "pleomorphic" sounds little appropriate to the *cytologic* picture of this tumor, made up by a *monomorphous* population of epithelium cells, in which a conspicuous admixture of overt mesenchymal cells is seldom noted.

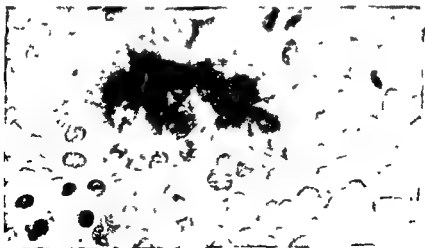


Fig 6

Pleomorphic adenoma. Tumor cells together with mucoid substance ( $\times 200$ )

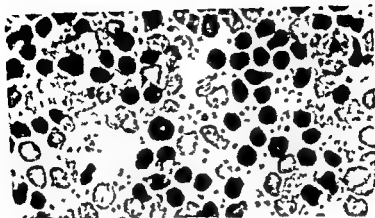


Fig 7

Detail of a parotid gland punctate from a case of Mikulicz disease. The smear has the character of a lymph node punctate dominated by lymphocytes, polymorphocytes and lymphoflasts.

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Giant cell of the Langhans type from a case of sarcoidosis (Parotid gland punctate  $\times 200$ )



rarer types of specific salivary gland tumors (Foote & Frazell 1953) were not represented in our material

A more or less conspicuous admixture of lymphocytes is often seen and suggests the presence of *chronic sialoadenitis*. In extreme cases the smears may be dominated by a complete lymphoglandular cytology, comprising reticular stem cells and sinus endothelium cells mixed with glandular epithelium cells (lymphoglandular metaplasia). Lymphatic infiltrations of the salivary glands is an interesting chapter which is still far from settled to judge from the confusing nomenclature ("lympho epithelial lesion", Mikulicz disease, Sjogrens syndrome etc.), (Zettergren 1959). The cytologic picture gives the impression of different degrees of one and the same lesion and doesn't permit a detailed differentiation. In addition it is wise to be cautious with the diagnostic conclusions: the punctates may be contaminated by lymphoglandular cells from separate lymph nodes lying close to the salivary gland (Fig 7).

*Epitheloid cell granulomas* are often clearly documented by the presence of epitheloid cells or giant cells of the Langhans' type. The typical marginal position of the nuclei is often lost in the smears (Fig 8). In most cases this finding means a confirmation of a clinical suspicion of sarcoidosis (uveo-parotid fever, Heerfordt's syndrome), for the differential diagnosis against tuberculosis a staining for tubercle bacilli is, of course, necessary.

The diagnosis of *cystic changes* is based on the macroscopic appearance of the punctate but also on the presence of large, free cyst phagocytes which may be similar to the glandular epithelium cells. They differ, however, by their character of "free" cells with distinct cell-borders (isolated gland cells occurring in the smears usually have the indistinct, ragged cellborders of "tissue-bound" cells). Cystic changes usually represent the conditions labelled *sialectasis* (Thackray 1955, Diamant 1958, 1959), a heterogeneous condition which is often met with in this type of diagnostic work.

The massive gain of *neutrophilic leucocytes and tissue phagocytes* met with in *acute sialoadenitis* invariably permits a reliable diagnosis of this condition.

## DIAGNOSTIC RESULTS

The cytologic conclusions were checked by histopathologic diagnosis (made by the pathologist of another hospital) in 28 cases only, reported in Table 1. In the remaining 62 cases a surgical intervention after the puncture was deemed unnecessary by the surgeon or refused by the patient. It must not be forgotten that, normally, a conventional biopsy had probably not been resorted to as a routine diagnostic measure in most of these cases. Some figures of Table 1 demand further comments.

A definite cytologic diagnosis of *carcinoma* was made in 2 cases and confirmed by histopathology. In 2 other cases the cytologic picture gave rise to a suspicion of malignant tumor, in one of these cases the eventual histologic diagnosis was cancer, in the other case not even the histologic diagnosis permitted more than a suspicion of cancer. In a third case in which a proliferation of reticular stem cells was the basis for a cytologic suspicion of malignant reticulosis, the final histologic diagnosis was epitheloid cell granuloma.

TABLE 1

Pathological/anatomical diagnosis	Cytol diagnosis						
	Cancer	Malignant tumor suspected	Pleomorphic adenoma	Epitheloid cell granuloma	Chronic parotitis	Normal parotitis	Inconclusive cytology
Cancer	2	1					
Malignant tumor suspected		1					
Pleomorphic adenoma (mixed tumour)			10				2
Epitheloid cell granuloma		1		5			
Chronic parotitis					1		1
Inconclusive histology						1	2
Normal parotitis							1

The cytologic diagnosis of *pleomorphic adenoma* was made in 12 cases. Histopathologic confirmation was obtained in 10 cases, in the remaining 2 cases the cytologic findings were typical, but no surgical intervention was made. The diagnosis of pleomorphic adenoma was missed in 2 cases, in which the cytologic findings were inconclusive.

In 5 cases the cytologic diagnosis of *epitheloid cell granuloma* was confirmed by histologic biopsy. In another 2 cases epitheloid cells were found, but histologic control was not obtained and the clinical data did not permit a definite clinical diagnosis.

The remaining cases, including the cases in which a histopathologic control was not obtained, represent miscellaneous conditions. In some cases the punctates permitted more or less definite

rarer types of specific salivary gland tumors (Foote & Frazell 1953) were not represented in our material

A more or less conspicuous admixture of lymphocytes is often seen and suggests the presence of *chronic sialoadenitis*. In extreme cases the smears may be dominated by a complete lymphoglandular cytology, comprising reticular stem cells and sinus endothelium cells mixed with glandular epithelium cells (lymphoglandular metaplasia). Lymphatic infiltrations of the salivary glands is an interesting chapter which is still far from settled to judge from the confusing nomenclature ("lympho-epithelial lesion", Mikulicz disease, Sjogrens syndrome etc.), (Zettergren 1959). The cytologic picture gives the impression of different degrees of one and the same lesion and doesn't permit a detailed differentiation. In addition it is wise to be cautious with the diagnostic conclusions: the punctates may be contaminated by lymphoglandular cells from separate lymph nodes lying close to the salivary gland (Fig 7).

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## DISCUSSION

The main value of fine-needle biopsy in the field of salivary gland diseases lies in the fact that it permits a reasonably accurate diagnosis of some specific lesions (*e g* cancer, pleomorphic adenoma, sarcoidosis) as a result of an extremely simple and innocuous intervention. Appropriate *therapeutic* measures may be based on such a positive cytologic diagnosis alone.

The finding of cystic changes or lymphocytic infiltration of the gland tissue, is, of course, much less informative, confronted with other features of the clinical picture even such findings may, however, contribute in a valuable way to the diagnosis. It is important to realize, however, that information of this type is not to compare with a pathological-anatomical diagnosis. Only positive diagnostic findings are really conclusive, negative findings don't exclude the presence of specific lesions. The limitations of the cytologic diagnosis must be clearly pointed out to the clinician. To get the best out of the method a close personal contact between clinician and cytologist is desirable, if possible the cytologist should make the punctures himself. If the gain of cells was insufficient in a first punctate he should not hesitate to secure a better material in a second puncture, in our experience no patient ever objected against a second puncture of this type.

The clinical picture and the subsequent course of the disease generally confirmed the practical value also of less specific types of cyto-diagnostic conclusions. In spite of the fact that they often had to be formulated in cautious, not to say vague terms, they often proved to offer important information as a complement to other clinical findings. There are still many obscure points in the pathology of the salivary glands, also the histologic diagnosis is difficult and often of restricted value in the clinical work. It may be that information gained by cytologic biopsy is often very fragmentary, but if this is duly realized, such fragments of information may be valuable building-stones in the final diagnosis.

## SUMMARY

The authors report personal experiences with fine-needle cytologic biopsy in diseases of the salivary glands. The method is stated to be technically simple and free of inconveniences and risks for the patient. It permits an accurate positive diagnosis of specific lesions such as pleomorphic adenoma, cancer and epitheloid cell granulomas. In other cases a peremptory diagnostic conclusion is often not possible, but the information gained may nevertheless be of decisive clinical value (lymphocytic infiltration, cystic changes etc.). As a whole the method must be regarded as a *diagnostic adjunct of considerable value*.

female animals. These were each given 0.1 ml of the tumour ascites intraperitoneally (tumour cell count  $1.960.000 \text{ mm}^3$ , tumour blood content—3 per cent).

Six days after the injection of the tumour all the mice were killed. One ml of the tumour ascites was centrifuged in a Wintrobe tube to obtain the PCA of the erythrocytes (Hartnell 1961) and the cell free supernatant ascitic fluid. This supernatant was then added to the original tumour as in the above experiment and films were made in the same way. The films were numbered 1-5 according to the amount of excess ascitic fluid used (Table 1). The end point of the reaction was recorded as the number of the tube in which all the tumour cells were either swollen or pyknotic, i.e. at which no healthy cells remained.

TABLE 1

*The Proportions of Whole Ehrlich Ascites Carcinoma in Cell Free Ascitic Fluid from the Same Mouse Used in the in vitro Tests*

Tube number	Whole tumour, drops	Ascitic fluid drops
1	1	0
2	2	1
3	1	1
4	1	2
5	1	4

## RESULTS

*Experiment I* Figure 1 shows the result of adding different amounts of ascitic fluid from the same tumour to the tumour cells (1a). The addition of small amounts of excess fluid resulted in the production of large injured cells (1b), greater amounts produced pyknosis (1c) and still greater amounts clumping (1d). The pyknotic cells were seen to be joined by fine bridges (Fig. 2a). These bridges were also seen on dark ground illumination of the wet preparation (2b). These findings were constant in all the mice investigated.

TABLE 2

*The Amount of Excess Ascitic Cells—Expressed as the Mean  $\pm$  (SD) of the Sex Difference*

Series	No. of mice	Mean	SD	SF	t	P
Blood content (%)						
♂	14*	2.5	1.9	0.88	1.46	$0.2 > P > 0.1$
♀	14*	1.2	1.6			
♂ + ♀	28	1.8	1.9	0.33	1.58	$0.2 > P > 0.1$
Tube number						
♂	14*	1.7	0.9			
♀	14*	2.2	0.9			
♂ + ♀	28	2.0	0.9			

\* Tumour unsuitable for investigation in one mouse

*Experiment II* Table 2 shows the mean blood content of the tumours, i.e. the PCV of the erythrocytes (with the standard deviation, SD) and

## THE IN VITRO DEMONSTRATION OF A CYTOTOXIC FACTOR IN EHRlich'S ASCITES CARCINOMA

By

F. HARTVEIT

Received 14 June 62

In a previous experiment (Hartveit 1962) the author found that the number of injured cells present in untreated Ehrlich ascites carcinoma at 6 days increases with the blood content of the tumour. When the blood content is particularly high the number is less than would be expected from the regression line. This finding, which has not been stressed previously, prompted the re-examination of the films from the tumours in the previous experiment. It was found that in the cases in which the number of injured cells was lower than expected many pyknotic cells were also present, while there were few in tumours with a low blood content.

In view of these findings, which suggest that there is a factor injurious to the tumour cells—a cytotoxic factor—in the ascitic fluid, it was decided to see if an excess of fluid over that present *in vivo* would increase the amount of cell damage produced (experiment I), and if this were so to see whether the different fluids varied in their ability to cause cell damage, and whether this ability was related to the tumour blood content (experiment II).

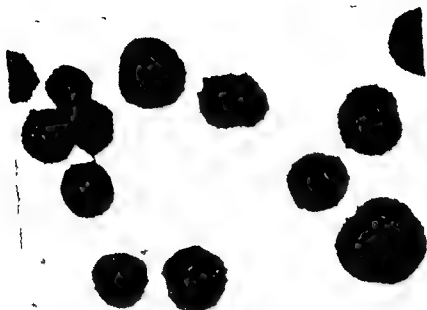
### MATERIAL AND METHODS

The mice and the Ehrlich ascites carcinoma used were similar to those used in previous experiments (Hartveit 1961) the tumour now being in its 132nd transplant generation.

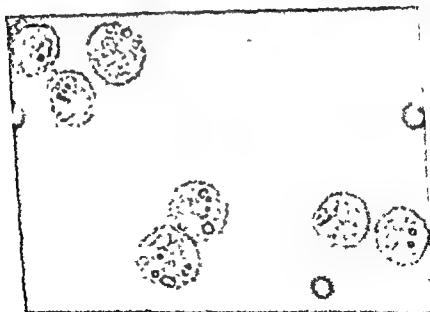
#### *Experimental Procedure*

**Experiment I** One mouse provided the tumour for the experimental group which consisted of 5 male and 5 female animals that were each given 0.1 ml of the tumour ascites intraperitoneally (tumour cell count 1 370 000/mm<sup>3</sup> tumour blood content —2 per cent). After 11 days the mice were killed and the tumour ascites removed. Half of the tumour ascites from each mouse was centrifuged to obtain the cell free ascitic fluid. This supernatant was then added to the other half of the tumour ascites using Pasteur pipettes in the proportions shown in Table 1. Films were made as described previously (Hartveit 1962) from the original tumours and from all dilutions.

**Experiment II** (Based on the results of experiment I *vide infra*) One mouse provided the tumour for this experimental group which consisted of 15 male and 15



a Leishman's stain  $\times 1400$



b Unstained wet preparation, dark ground illumination,  $\times 1100$

Fig 2

Bridge formation between pyknotic Ehrlich ascites carcinoma cells following the addition of an excess of cell free ascitic fluid from the same tumour to the unwashed cells

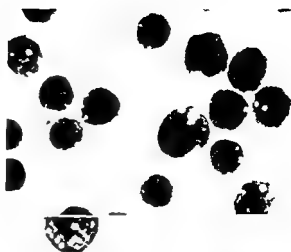


*Fig 1*

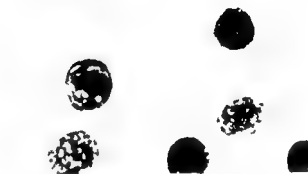
The effect of an excess of  
ascitic fluid from the same  
tumour on Ehrlich ascites  
carcinoma cells

Leishman's stain  $\times 720$   
(reduced to approx  
 $\times 580$  in print)

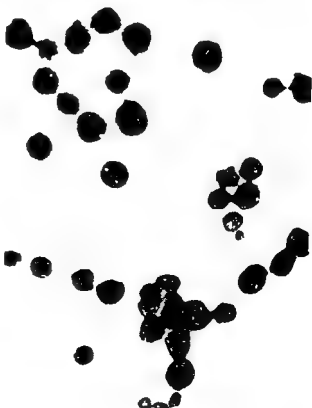
- a Whole tumour  
Note normal cell size



- b 1 drop of tumour plus 1 drop  
of ascitic fluid Note swollen  
cells and loss of cytoplasm

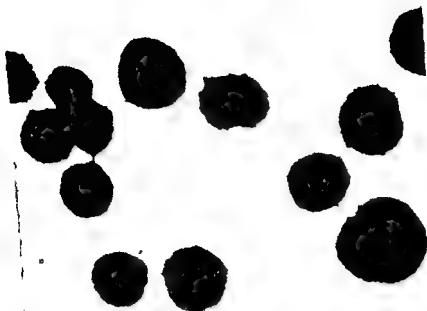


- c 1 drop of tumour plus  
2 drops of ascitic fluid  
Note pyknosis

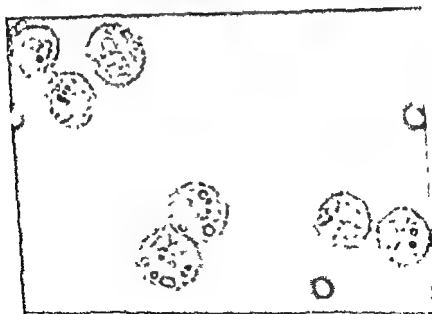


- d 1 drop of tumour plus  
4 drops of ascitic fluid  
Note pyknosis and clumping





a Leishman's stain  $\times 1400$



b Unstained wet preparation dark ground illumination  $\times 1100$

Fig 3

Bridge formation between pyknotic Ehrlich ascites carcinoma cells following the addition of an excess of cell free ascitic fluid from the same tumour to the unwashed cells

*Fig 1*

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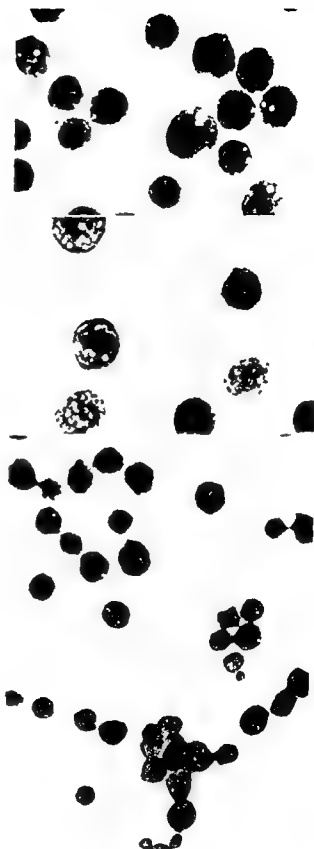
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a Whole tumour  
Note normal cell size

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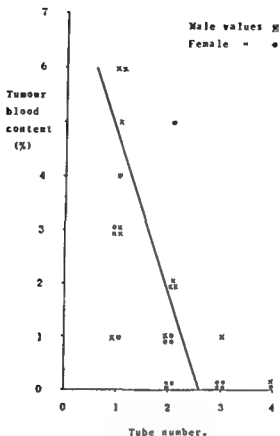


Fig 3

Regression line and scatter diagram of the relationship between the tumour blood content and the amount of excess tumour ascitic fluid needed to injure all the tumour cells—expressed as the tube number (see text), for the total series

the mean amount of excess ascitic fluid needed to produce changes in all the tumour cells (SD) for the male, female and total series. The standard error (SE) of the difference between the male and female means is also given, with the  $t$  and  $P$  values.

Table 2 shows that the sex difference was not significant for either factor. Therefore the total series was used for the regression line (Fig 3) of the relationship between the tumour blood content ( $x$ ) and the amount of excess ascitic fluid needed to produce changes in all the tumour cells ( $y$ ), ( $y = 2.58 - 0.33x$ ). The corresponding correlation coefficient,  $r$ , was found to be 0.6934 ( $t = 3.602$ ) and as such was highly significant ( $0.01 > P > 0.001$ ) ( $r^2 = -0.6766$ ,  $r^2 = -0.6517$ ).

## DISCUSSION

The hypothesis on which the present experiments are based is that there is a cytotoxic factor in the ascitic fluid surrounding the Ehrlich ascites carcinoma cells. A previous experiment (Harvell 1962) had suggested that in some cases enough of this factor is present *in vivo* to

damage the tumour cells, and that the amount of damage varied from tumour to tumour. Experiment I was therefore designed to see if the addition of ascitic fluid in excess of that found *in vivo* would increase the cell damage. The results show that it did, small amounts producing the type of injury, seen *in vivo*, that has been shown to be related to the tumour blood content (Hartvelt 1962), larger amounts producing pyknosis and clumping of the tumour cells. The formation of bridges between the pyknotic cells preceded clumping. As these bridges were seen in the wet preparations they are not fixation artefacts. The phenomenon is not purely mechanical as it occurred with different proportions of cells and ascitic fluid in different tumours (*vide infra*).

Direct reference to bridges of this type between tumour cells has not been found, but such a bridge has been clearly demonstrated in a photograph in Fisch's work (1962) on this tumour. This photograph (Fisch's Fig. 24) shows the result of treating unwashed tumour cells with specific fluorescent labelled anti-tumour globulin absorbed with mouse liver powder. Bridges have also been shown to be present between erythrocytes when they have been agglutinated by specific antibody (Stratton & Renton 1958). Thus this finding adds further support to the idea (Hartvelt 1962) that an immunological reaction is responsible for the cell damage.

Experiment II was designed to see if the amount of this factor varies in the different ascitic fluids, and whether it is related to the tumour blood content, as suggested from the findings in a previous experiment (Hartvelt 1962). It was found that the different fluids did vary (Fig. 3) in their ability to injure the cells of the tumour from which they were taken. It was also clearly shown that this ability is related to the tumour blood content—a fluid from a tumour with a high blood content being more active than one from a non haemorrhagic tumour. The considerable scatter in the results in Figure 3 may well be due to the experimental error inherent in the use of Pasteur pipettes, as the drop size was not uniform, different pipettes being used for tumour and ascitic fluid.

The results of these experiments provide further evidence of the existence of a cytotoxic factor in untreated Ehrlich ascites carcinoma, and suggest that this factor is related to the immune reaction to the homografted tumour that is reflected in the tumour blood content (Hartvelt 1961). There are two possible explanations of the findings. The cytotoxic factor may be present in the ascitic fluid that is added to the whole tumour. In this case an excess of the factor in relation to the cells is usually needed. The second possibility is that the factor is present on the unwashed cells and that an additional factor, present in the ascitic fluid, is needed in excess to trigger off the reaction. In this connection it is of note that the interaction of Ehrlich ascites carcinoma cells and immune antiserum from a foreign host has been shown to take place only when complement has been added to the sy-

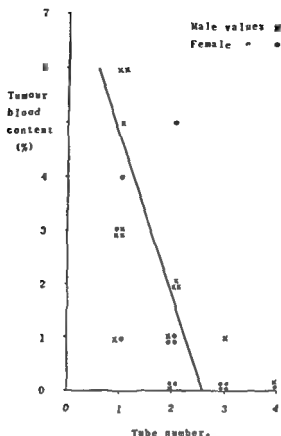


Fig 3

Regression line and scatter diagram of the relationship between the tumour blood content and the amount of excess tumour ascitic fluid needed to injure all the tumour cells—expressed as the tube number (see text), for the total series

the mean amount of excess ascitic fluid needed to produce changes in all the tumour cells (SD) for the male, female and total series. The standard error (SE) of the difference between the male and female means is also given, with the *t* and *P* values.

Table 2 shows that the sex difference was not significant for either factor. Therefore the total series was used for the regression line (Fig 3) of the relationship between the tumour blood content (*x*) and the amount of excess ascitic fluid needed to produce changes in all the tumour cells (*y*), ( $y = 2.58 - 0.33x$ ). The corresponding correlation coefficient, *r*, was found to be 0.6934 (*t* = 3.602) and as such was highly significant ( $0.01 > P > 0.001$ ) ( $r_s = -0.6766$ ,  $r_p = -0.6517$ ).

## DISCUSSION

The hypothesis on which the present experiments are based is that there is a cytotoxic factor in the ascitic fluid surrounding the Ehrlich ascites carcinoma cells. A previous experiment (Hartveit 1962) had suggested that in some cases enough of this factor is present *in vivo* to

damage the tumour cells, and that the amount of damage varied from tumour to tumour. Experiment I was therefore designed to see if the addition of ascitic fluid in excess of that found *in vivo* would increase the cell damage. The results show that it did, small amounts producing the type of injury, seen *in vivo*, that has been shown to be related to the tumour blood content (Hartveit 1962), larger amounts producing pyknosis and clumping of the tumour cells. The formation of bridges between the pyknotic cells preceded clumping. As these bridges were seen in the wet preparations they are not fixation artefacts. The phenomenon is not purely mechanical as it occurred with different proportions of cells and ascitic fluid in different tumours (*vide infra*).

Direct reference to bridges of this type between tumour cells has not been found, but such a bridge has been clearly demonstrated in a photograph in Fitch's work (1962) on this tumour. This photograph (Fitch's Fig. 24) shows the result of treating unwashed tumour cells with specific fluorescent labelled anti tumour globulin absorbed with mouse liver powder. Bridges have also been shown to be present between erythrocytes when they have been agglutinated by specific antibody (Stratton & Renion 1958). Thus this finding adds further support to the idea (Hartveit 1962) that an immunological reaction is responsible for the cell damage.

Experiment II was designed to see if the amount of this factor varies in the different ascitic fluids, and whether it is related to the tumour blood content as suggested from the findings in a previous experiment (Hartveit 1962). It was found that the different fluids did vary (Fig. 3) in their ability to injure the cells of the tumour from which they were taken. It was also clearly shown that this ability is related to the tumour blood content—a fluid from a tumour with a high blood content being more active than one from a non-haemorrhagic tumour. The considerable scatter in the results in Figure 3 may well be due to the experimental error inherent in the use of Pasteur pipettes, as the drop size was not uniform, different pipettes being used for tumour and ascitic fluid.

The results of these experiments provide further evidence of the existence of a cytotoxic factor in untreated Ehrlich ascites carcinoma, and suggest that this factor is related to the immune reaction to the homografted tumour that is reflected in the tumour blood content (Hartveit 1961). There are two possible explanations of the findings. The cytotoxic factor may be present in the ascitic fluid that is added to the whole tumour. In this case an excess of the factor in relation to the cells is usually needed. The second possibility is that the factor is present on the unwashed cells and that an additional factor, present in the ascitic fluid, is needed in excess to trigger off the reaction. In this connection it is of note that the interaction of Ehrlich ascites carcinoma cells and immune antiserum from a foreign host has been shown to take place only when complement has been added to the sy-



stem (Flax 1956, Wissler & Flax 1957, Fitch 1962, Stone Dzoga & Wissler 1962) Similarly, with the Yoshida ascites sarcoma complement was needed to produce the *in vitro* changes in the tumour cells (Lund 1957) Further experiments on the mode of action of the cytotoxic factor in Ehrlich's ascites carcinoma are in progress

### SUMMARY

The presence of a factor in untreated Ehrlich ascites carcinoma that is capable of damaging the tumour cells *in vitro* is demonstrated The morphology of the injured cells and the formation of bridges between the pyknotic cells suggest that an immunological reaction is involved This is also in accordance with the finding that the activity of the tumours in this respect is proportional to their blood content, a measure of the immune reaction of the host to the tumour homograft (Hartveit 1961)

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## THE VIABILITY OF PYKNOTIC EHRLICH ASCITES CARCINOMA CELLS

By

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In describing the stages in the death of an Ehrlich ascites carcinoma cell King, Paulson, Puckett & Krebs (1959) have shown that the cell bursts before it becomes pyknotic. The findings in autolytic tumour support this view (Hartveit 1962b). The author has reported previously (Hartveit 1963) that Ehrlich ascites carcinoma cells treated with an excess of ascitic fluid from the same tumour become swollen, if the amount of excess fluid is low, and pyknotic if the amount is greater. The latter cells are obviously damaged but may or may not be dead. To settle this point the survival time of mice after the intraperitoneal injection of undiluted tumour, tumour diluted in physiological saline and tumour diluted with cell free tumour ascitic fluid was studied.

### MATERIAL AND METHODS

The mice and the Ehrlich ascites carcinoma used were similar to those used in previous experiments (Hartveit 1961) the tumour now being in its 130th transplant generation.

**Experimental procedure.** Five male mice that had received 0.1 ml of cell

proliferated and the supernatant ascitic fluid

male and 10 female mice were set up  
intraperitoneal injection of 0.1 ml of the  
the same amount of tumour ascites  
logical saline and group III the same  
with pooled cell free tumour ascitic

**Investigations.** Films were made as described previously (Hartveit 1962b) from the tumour injected in each group. Wet mounts were

*Fig 1*

The Ehrlich ascites carcinoma cells injected in the 3 groups  
 Dark ground illumination  $\times 560$   
 (reduced to approx  $\times 450$  in print)

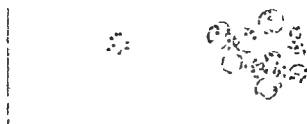
a Group I  
 Note healthy cells

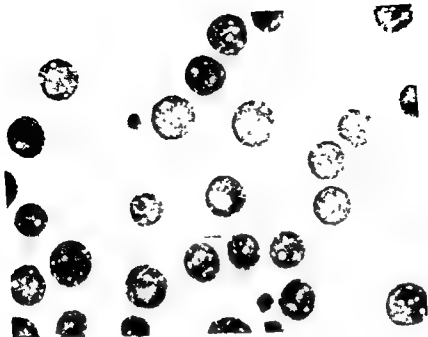


b Group II  
 Note slight shrinkage



c Group III  
 Note pyknosis and clumping





a Group I Note normal cell size



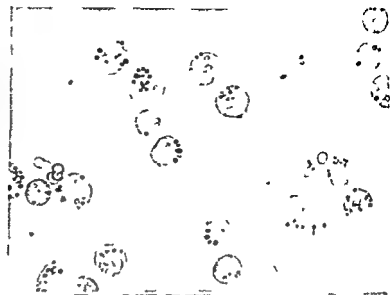
b Group III Note pyknosis and clumping

Fig 2

The Ehrlich ascites carcinoma cells injected in group I, undiluted, and in group III, diluted with ascitic fluid. Leishman's stain,  $\times 700$

*Fig 1*

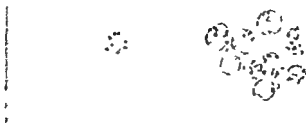
The Ehrlich ascites carcinoma cells injected in the 3 groups  
 Dark ground illumination  $\times 560$   
 (reduced to approx  $\times 450$  in print)



a Group I  
 Note healthy cells



b Group II  
 Note slight shrinkage



c Group III  
 Note pyknosis and clumping

TABLE 2

The Survival Time ( $\bar{x}$ ) in Days (SD) of the Mice in the 3 Groups (see Table 1 a)  
The Sex Differences within the Groups with SF  $t$  and  $P$  Values Are Given  
(10 Male and 10 Female Mice in each Group)

Group	Series	$\bar{x}$	SD $\bar{x}$	Sex diff $t$	SF	$t$	$P$
I	♂	13.5	2.4	15	0.93	1.61	$0.2 > P > 0.1$
	♀	15.0	2.2				
II	♂	15.4	2.7	18	1.24	1.45	
	♀	13.6	2.8				
III	♂	14.4	1.9	23	1.05	2.12	$0.05 > P > 0.02$
	♀	16.7	2.8				

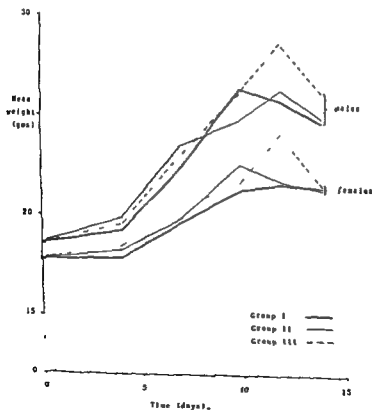


Fig 3

The mean weight of the mice in the different groups

## RESULTS

Figure 1 shows wet preparations of the tumour injected in each of the experimental groups. The tumour cells in Figure 1a—group I, injected undiluted—appear healthy. Some of those in Figure 1b—group II, diluted with saline—are slightly shrunken. The cells in Figure 1c—group III, diluted with ascitic fluid—are pyknotic and show clumping. This pyknosis is further evident in Figure 2 which shows the undiluted cells (2a) and the cells diluted in ascitic fluid (2b). The pyknosis of the cells in the latter fluid was uniform, no healthy cells remaining. The effect of diluting with pooled ascitic fluids appeared identical to that previously seen on adding an excess of fluid from the same tumour (Hartwell 1963).

TABLE 1a

*The Diameter ( $\bar{x}$ ) of the Ehrlich Ascites Carcinoma Cells in the 3 Groups I—Guen Undiluted Ehrlich Ascites Carcinoma II—Tumour Diluted with Saline and III—Tumour Diluted with Ascitic Fluid as Measured from Photographs*

Group	$\bar{x}$ (mm)	SD $\bar{x}$
I	9.16	1.41
II	8.38	1.17
III	6.56	0.73

TABLE 1b

*The Difference in Cell Diameter ( $\bar{x}$ ) between the 3 Groups (see Table 1a) with the SE,  $t$  and  $P$  Values*

Difference between groups	Diff in $\bar{x}$ (mm)	SE	$t$	$P$
I and II	0.78	0.41	1.77	$0.1 > P > 0.05$
II and III	1.82	0.69	2.64	$0.02 > P > 0.01$
I and III	2.60	0.39	6.72	$0.001 > P$

Table 1a gives the mean cell diameter, with standard deviation (SD) of the cells injected in each group, and shows that slight shrinkage occurred in cells diluted with saline and marked shrinkage in those diluted with ascitic fluid. Table 1b shows the differences in cell diameter between the groups, with the standard error (SE),  $t$  and  $P$  values. The amount of shrinkage in the saline diluted cells is not significant, while that of those diluted with ascitic fluid is highly so ( $0.001 > P$ ).

Table 2 gives the survival time (SD) of the mice in all three groups and the sex differences within the groups, with SE,  $t$  and  $P$  values. The sex difference was only significant in group III ( $0.05 > P > 0.02$ ).

TABLE 2

The Survival Time ( $x$ ) in Days (SD) of the Mice in the 3 Groups (see Table 1 a)  
The Sex Differences within the Groups with SF  $t$  and  $P$  Values Are Given  
(10 Male and 10 Female Mice in each Group)

Group	Sexes	$x$	SD $x$	Sex diff $t$	$M$	$t$	$P$
I	♂	13.5	2.4	1.5	0.93	1.61	$0.2 > P > 0.1$
	♀	15.0	2.2				
II	♂	15.4	2.7	1.8	1.24	1.45	
	♀	13.6	2.8				
III	♂	14.4	1.9	2.3	1.09	2.12	$0.05 > P > 0.02$
	♀	16.7	2.8				

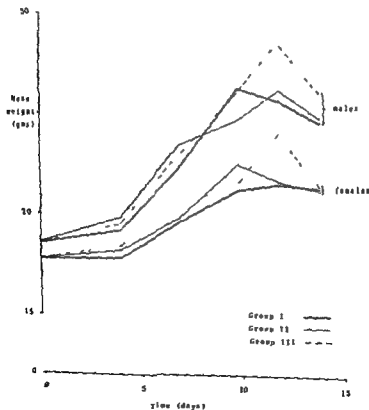


Fig 3

The mean weight of the mice in the different groups



Table 3 gives the difference in survival time between the groups, with SE, *t* and *P* values. The difference between the females in groups II and III was significant ( $0.05 > P > 0.02$ )

TABLE 3

*The Difference in Survival Time ( $\tau$ ) in Days between the 3 groups (see Table 1a) with the SE, *t* and *P* Values (10 Male and 10 Female Mice in each Group)*

Difference between groups	Series	Sex diff $\tau$	SE	<i>t</i>	<i>P</i>
I and II	♂	1.9	1.15	1.65	$0.2 > P > 0.1$
	♀	1.4	1.00	1.39	
II and III	♂	1.0	0.62	1.61	$0.05 > P > 0.02$
	♀	3.1	1.26	2.47	
I and III	♂	0.9	0.97	0.93	$0.4 > P > 0.3$
	♀	1.7	1.05	1.62	

On vital staining of the tumour cells in the three fluids injected none of the tumour cells took up the vital stain at 2 minutes, at 15 minutes or at 45 minutes, at which time all the injections had been completed. After 24 hours 7 per cent of the cells in the group I fluid, 10 per cent in group II, and 50 per cent in the group III fluid took up the vital stain.

Figure 3 shows the weights of the mice in the 3 groups related to time. The differences between the groups are not statistically significant.

## DISCUSSION

The results of this experiment show that although morphological signs of injury can be seen in Ehrlich ascites carcinoma cells treated with an excess of tumour ascitic fluid the cells are not dead. This is in keeping with the results of vital staining but not in keeping with the idea that the pyknotic tumour cells are cells that have burst. King, Paulson, Puckett & Krebs (1959) have shown that damaged tumour cells swell, burst and subsequently become pyknotic and take up the vital stain. The author found the same in cells undergoing non-specific autolysis (Hartveit 1962b). It has also been shown that when a small amount of excess ascitic fluid is added to the tumour cells swelling results, but when a large amount of fluid is added all the cells become pyknotic (Hartveit 1963), preliminary swelling has not been observed.

This suggests that the injury inflicted by a great excess of the fluid differs from the primarily cytoplasmic injury that follows a smaller amount of the same fluid, in that the cell does not burst. This idea is supported by the findings in the present experiment. Vital staining showed that the tumour cells that had been diluted with ascitic fluid

did not take up the stain after 2 minutes—as they should have done had they been burst cells (King Paulson Puckett & Krebs 1959). After 2 hours half of them did so—in contrast to the controls—confirming that some damage was present.

The survival time of the mice in the present experiment clearly indicates that the pyknotic tumour cells were not dead (Table 3—the difference in survival time between the untreated cells (group I) and those diluted with ascitic fluid (group III) was not significant. The weight curves for the mice in the different groups (Fig. 3) show that there was no preliminary lag in group III that could indicate that a lower dose of living cells had been compensated by an AIT effect exerted by the dead cells present.

The changes in cell diameter and morphology (Figs. 1 and 2 and Table 1a) suggest that the cells in group III are damaged. This is supported by the results of the vital staining and of the survival time experiments in the females (vide infra). It has been shown previously that female mice have greater natural resistance to Ehrlich's ascites carcinoma than males (Hartveit 1962a). This does not usually show up on intraperitoneal injection. But in group III of this experiment the females survived significantly longer than the males ( $0.05 > P > 0.02$ ) (Table 2). This finding suggests that the cells were damaged so that they were more vulnerable than usual to the immune response of the host. This is further supported by the finding (Table 3) that the females in group III also survived significantly longer than those in group II ( $0.05 > P > 0.02$ ). The group II cells had been diluted in saline, i.e. they had gone through the same mechanical process as those in group III but their vitality was not impaired in contrast to that of the cells diluted in ascitic fluid.

It is of note that Ehrlich ascites carcinoma cells treated with heterologous immune gamma globulin show swelling. Whether or not rupture of the cells takes place is debated (Flax 1956; Green Barrow & Goldberg 1959) but their viability is definitely reduced (Lindner 1960). The changes in such cells appear to be identical to those following a small amount of excess ascitic fluid and the reduced viability is in keeping with the finding that the cells with the latter changes show vital staining after about 10 minutes (Hartveit 1962b) in contrast to the pyknotic cells in the present experiment that needed two hours.

It thus seems that the injury to the tumour cells following a great excess of ascitic fluid is reversible at least in the early stages while that following smaller amounts may not be. This possibility is being investigated.

#### SUMMARY

Ehrlich ascites carcinoma cells treated with an excess of tumour ascitic fluid become pyknotic. These pyknotic cells are not dead but injured. It is suggested that the pyknosis of the cells has not been pre-

ceded by cell rupture with consequent irreversible cell damage. The experiment also presents further evidence that female mice show greater natural immunity to the Ehrlich ascites carcinoma than male mice.

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## SEX DIFFERENCES IN THE INTRAPERITONEAL GROWTH OF EHRlich'S ASCITES CARCINOMA

By

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Previous experiments have indicated that there is a difference in the reaction of male and female mice to the transplantation of Ehrlich's ascites carcinoma. This difference does not appear to effect the survival time of the animals following intraperitoneal injection of the tumour (Hartveit 1961a) but it does show up on subcutaneous injection (Hartveit 1962a). It does not seem to be reflected in the blood content of the intraperitoneal tumour (Hartveit 1961a & b) but it does show up on the transplantation of pyknotic tumour cells (Hartveit 1963b). From these experiments it appears that female mice may possess more natural immunity to the homotransplant than males.

The following experiment was designed to study the morphology of the Ehrlich ascites carcinoma cells at various times after transplantation to see if this would give any further information on the above mentioned sex difference.

### MATERIAL AND METHODS

The mice and the Ehrlich ascites carcinoma used were similar to those used in previous experiments (Hartveit 1961a) the tumour now being in its 133rd transplant generation.

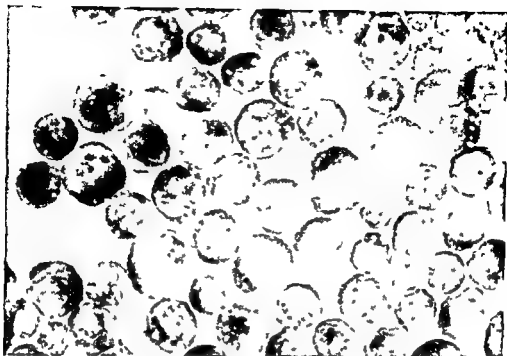
*Experimental procedure*

Serial biopsies were taken from these mice at three day intervals by inserting a needle through the abdominal wall and collecting the drop of tumour ascites that formed when the needle was withdrawn. Films were made from the tumour cells and stained by the method of Hartveit (1961a). The tumour blood content—1 per cent.

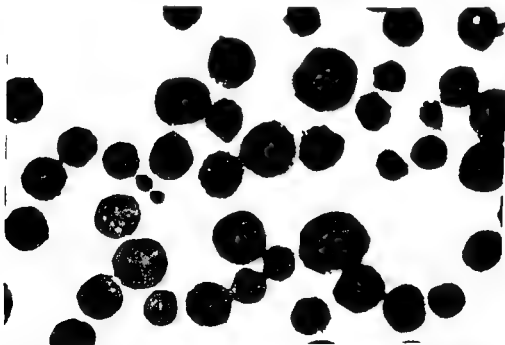
Serial biopsy specimens were taken from these mice at three day intervals by inserting a needle through the abdominal wall and collecting the drop of tumour ascites that formed when the needle was withdrawn. Films were made from the tumour cells and stained by the method of Hartveit (1961a). The tumour blood content—1 per cent.

### RESULTS

Four main types of Ehrlich ascites carcinoma cells were seen. These were the normal cells (Fig. 1a), pyknotic cells (Fig. 1b) and two types



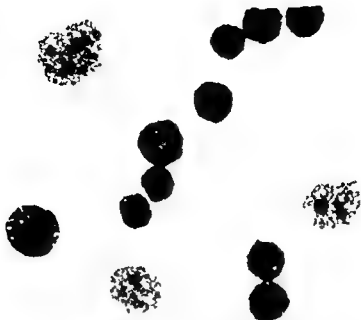
a Biopsy specimen of tumour 3 days after transplantation Normal cells  
Note size shape and stainability



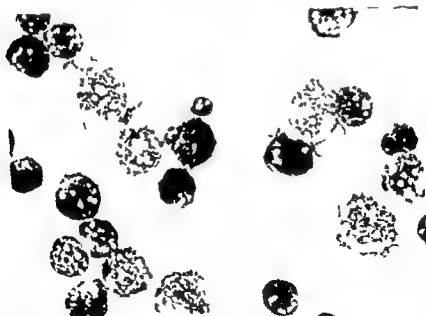
b Biopsy specimen of tumour from the same mouse 3 days after transplantation  
Note flattening of cell surface increase in stainability intercellular  
ridges and clumping

Fig 1

Changes in untreated Ehrlich ascites carcinoma cells following transplantation  
Leishman's stain  $\times 700$



a Type 1 from first biopsy showing pyknosis. Note disintegration of cytoplasm and presence of nucleoli



ii Type 2 from biopsy 9 days later. Note simultaneous disruption of cytoplasm and nucleus with loss of nucleoli. Also transition via lesser changes of same type from pyknotic to large cells

Fig 2

Large injured tumour cells in untreated Ehrlich ascites carcinoma,  
Leishman's stain  $\times 700$

of large injured tumour cell. The first of these (type 1) was similar to those described previously (Hartveit 1962b) that showed swelling and primary cytoplasmic damage indicative of immunological type damage (Fig 2a). The second type of large injured tumour cell (type 2) was clearly different from the type 1 cells in that the nuclear and cytoplasmic damage was seen to occur simultaneously and transition stages between these and the pyknotic tumour cells were seen (Fig 2b).

Normal and type 1 tumour cells were present in early biopsies. After a certain time, which varied from mouse to mouse, the majority of the tumour cells lost their previous spherical shape—their surface showed flat planes—and pyknosis occurred, with increased stainability. Inter-cellular bridges were also seen between these pyknotic cells and clumping was also present (Figs 1a and 1b). A few type 1 cells were still present in the first biopsy showing pyknosis but after this time the tumour was made up of pyknotic and type 2 cells.

Figure 3 shows the time of occurrence of pyknosis. There was a marked sex difference. Pyknosis tended to occur earlier in the tumours in female mice. It was present in 6 of these at 3 days while none of the males had pyknotic tumours at this time. This difference is statistically significant ( $0.001 > P$ ).

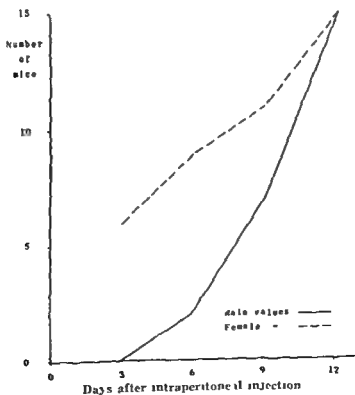


Fig 3

The time of occurrence of *in vivo* pyknosis in Ehrlich's ascites carcinoma (15 ♂ and 15 ♀ mice)

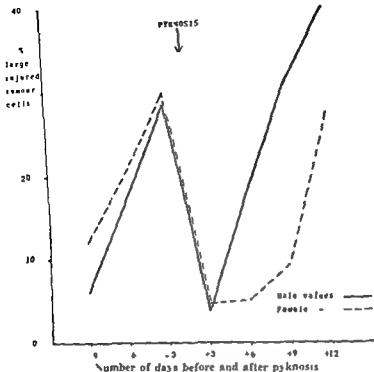


Fig. 4

The mean percentage of large injured Ehrlich ascites carcinoma cells present in biopsies taken before and after *in vivo* pyknosis of the tumour (see Table 1)

TABLE 1

The Percentage of Large Injured Tumour Cells (Types 1 & 2) in Untreated Ehrlich Ascites Carcinoma Related to the Time of *in vivo* Pyknosis of the Tumour Cells (Data from 15 ♂ & 15 ♀ Mice)

		Days before pyknosis*			Days after pyknosis*			
		9	6	3	3	6	9	12
Large injured tumour cells		Type 1			Type 2			
Mean %	♂	6.6	17.2	28.1	4.0	17.6	31.5	39.0
	♀	12.0	20.2	29.9	4.6	5.0	9.0	20.7
S.D.	♂	7.8	10.0	6.2	2.1	13.3	7.9	7.0
	♀	6.5	7.9	8.1	2.4	2.9	11.0	17.5
Number of biopsies	♂	8	13	11	13	9	6	2
	♀	35	55	9	15	14	12	8

\* Maximum time possible

‡ Biopsy unsuccessful in one other mouse



Table 1 shows the mean percentage of large damaged tumour cells, of both types, present in the biopsies preceding and following the occurrence of pyknosis. The standard deviation of these values and the number of biopsies studied is given. The results are shown graphically in Fig. 4.

In both sexes the number of injured tumour cells—type 1—rose to a peak in the biopsy preceding pyknosis and fell sharply when pyknosis had occurred. This drop in the number of type 1 cells is highly significant in both sexes ( $0.001 > P$ ). After this time a further sex difference is apparent. The number of type 2 cells rose more quickly in the males than in the females, the differences in the two biopsies following the first showing pyknosis (6 and 9) being significant ( $0.001 > P$ ).

### DISCUSSION

The results of serial biopsy in the present experiment in which the number of injured tumour cells was seen to increase with time, and to reach a peak which was followed by pyknosis of the cells, are reminiscent of the findings on adding an excess of ascitic fluid to the tumour cells (Hartveit 1963i). In this previous experiment it was seen that when few large injured cells were present *in vivo* the addition of excess ascitic fluid increased their numbers and an even greater excess resulted in the pyknosis of the cells. The large injured cells produced were of the type seen in early biopsies in the present experiment—type 1 cells. The addition of even greater amounts of excess ascitic fluid did not give rise to type 2 cells as were seen in the later biopsies.

This indicates that although an increase in the amount of a cytotoxic factor may be responsible for the injury seen in type 1 cells it is unlikely to be the cause of the injury seen in the type 2 cells. This idea is under investigation and is supported by the morphology of the type 2 cells, in which the injury appears to affect both nucleus and cytoplasm simultaneously. In addition, as shown in Fig. 2b, the majority of the cells show minor changes of a similar nature, changes that are more like those seen on *in vitro* autolysis (Hartveit 1962b) than those due to immunological damage. It is suggested that these cell changes are probably due to anoxia, particularly as they were not seen until later biopsies when the amount of ascites was large and the host moribund.

A striking finding was that the biopsy with the greatest number of type 1 cells was invariably followed by a biopsy containing only a few injured cells, the rest being pyknotic. These pyknotic cells are undoubtedly viable. Klein & Ruvetz (1953) found no difference in the amount of inoculum needed to produce an ascitic tumour from tumours between 3 and 16 days after transplantation. This is supported by the author's own experience that a 10 day tumour, in which the cells are usually pyknotic, is as reliable for transplantation as a 6 day tumour in which the cells are of normal appearance. In addition tumour cells

of similar pyknotic appearance produced by adding an excess of ascitic fluid to the tumour ascites are viable (*Hartvelt 1963b*)

It thus seems that after a critical percentage of injured tumour cells is present the remaining cells shrink and appear pyknotic. This critical percentage seems to be around 30 per cent and to be the same in both sexes. The day this peak is reached varies greatly (presumably due to phenotypic and genotypic variations in the mice) and here there is a marked sex difference: the peak and subsequent pyknosis occurring before the 3rd day in 6 of the females in contrast to the males (Fig. 2). This suggests that the immunological response occurs earlier in the females than in the males.

A further marked sex difference is that the number of large injured cells in the two biopsies following that showing pyknosis was lower in the females than in the males (Fig. 4). This is probably a reflection of the fact that pyknosis tended to occur earlier in the females and that the tumours were not so large by the time these biopsies were taken. Incidentally this further supports the idea that the type 2 changes are the result of anoxia.

Unfortunately the cytological findings cannot be related to the survival time in this experiment as serial biopsy in itself may interfere with the survival time. Similarly the blood content of the tumour (*Hartvelt 1961b*) cannot be measured reliably. However the findings show that the greater immunity of female mice to the subcutaneous injection of Ehrlich's ascites carcinoma (*Hartvelt 1962a*) is reflected in the morphology of the intraperitoneal tumour in that the results of the immune response (i.e. increase in the number of injured cells and pyknosis) are seen earlier in female mice.

While the time of occurrence of the immune response is different in the two sexes the mechanism of the response itself appears to be the same: there are no significant differences in values obtained up to and including the first biopsy showing pyknosis, and it is probable that the time factor is also responsible for the differences after this time (vide supra).

#### SUMMARY

The morphological changes in Ehrlich ascites carcinoma cells were followed by serial biopsy. The number of large injured cells was found to increase with time until a critical percentage (30 per cent) was reached. Thereafter there were a few large injured tumour cells while the rest were pyknotic. Large injured tumour cells reappeared in later biopsies but were of different morphology from those preceding pyknosis. It seems likely that the injury in the latter cells is due to immunological damage while that in the former may well be the result of anoxia.

There was a marked sex difference in the mode of growth of the tumour in that pyknosis occurred earlier in the females. This supports

the author's previous findings (Hartveit 1962a and 1963a) that female mice have greater natural immunity to Ehrlich's ascites carcinoma than males

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## WHITE BLOOD CELLS FROM ANIMALS WITH EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS TESTED ON GLIA CELLS IN TISSUE CULTURE

By

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The experimental allergic encephalomyelitis (EAE) that can be induced in various species by immunization with brain or spinal cord material, together with Freund's complete adjuvant, is usually supposed to be caused by a delayed type of hypersensitivity. The lymph cells are generally regarded as mediators of the disease. Passive transfer with immune serum has failed but transfer with cell suspensions from lymph nodes of immunized rabbits, guinea pigs, and rats has been successful. Reviews on these problems are given by Waksman (1959) and Chase (1959), and more recent studies on cellular transfer of the disease are presented by, among others, Paterson (1960), Stone (1961), and Åström & Waksman (1962).

The importance of circulating serum antibodies, however, was stressed in recent studies by Bornstein *et al.* (1961, 1962) and the present authors (1962a). It was shown that sera from animals with EAE damaged glia cells and myelin in tissue cultures prepared from neonatal rat brains. Further interest in this phenomenon was evoked by the observations (Berg & Kallén 1961, 1962b, Bornstein 1962) that similar factors were found in sera from patients with neurological diseases, among these multiple sclerosis. Among other things the negative results of passive transfer of serum at EAE argue against a pathogenetic importance of serum factors.

In the present study, a toxic effect of white blood cells from rabbits with EAE on neonatal rat glia cells, cultured on reconstituted rat tail collagen, is described. Goverts (1960) showed that

... by Stuart (1962). Sharp & Burwell (1962) extensively discussed this literature and also presented evidence for a growth arresting effect of sensitized lymph cells on fibroblastic growth from spleen tissue. In most of these studies, complement was

not added to the test systems Klein & Sjogren (1960) incubated *in vitro* tumour cells with lymph cells immunized against the tumour cells, and showed that a destruction of the tumour cells occurred. No complement was present.

Since, the preparation of the present paper, a very similar study has appeared, written by Koprowski & Fernandes (1962), describing a "contactual agglutination" of sensitized lymph node cells from LAE animals in brain tissue culture, accompanied by destruction of glial elements.

## MATERIAL AND METHODS

Rabbits and rats were used as experimental animals. The rabbits were of mixed stock supplied commercially. The rats belonged to the homozygous Amsterdam R strain.

Immunization was performed with porcine spinal cord and Freund's complete adjuvant (Difco). The rabbits received injections into one foot pad of each of three legs the intradermal doses injected being 0.05 ml. Rats received 5-6 dorsal intradermal injections of each 0.05-0.1 ml. 6 rabbits were injected with pig kidney tissue and Freund's complete adjuvant.

At various times after immunization blood was collected from the rabbits usually by carotid bleeding. The blood was allowed to flow freely into a test tube containing 6 per cent dextran (Macrodex, Pharmacia) and a few drops of heparin. The proportions of dextran and blood was roughly 1:3. The blood was left to sediment and the clear fluid containing most of the white blood cells and some red cells was removed after 15-45 minutes. The cell suspension was centrifuged at approx. 1000 r.p.m. washed with a large amount of Parker 199 medium, spun down again and re-suspended in Parker 199. A cell count of 2.5 millions/ml was obtained. This suspension was added to the cultures.

In a few rabbits, the thoracic duct was cannulated. This operation was performed in morphine urethane anaesthesia. After removal of the first rib on the left side of the animal a thin plastic cannule was inserted into the duct and the lymph which flowed freely was collected in a test tube containing a small amount of heparin in Parker 199. The cells were spun down and washed as described for the blood cells and a suspension of 3-4 millions/ml was prepared in Parker 199. Practically all cells obtained in this way are lymphocytes.

The rats were cannulated through the thoracic duct using the method described by Saldeen & Linder (1960). These cells too were washed and re-suspended.

Glia cultures, prepared from neonatal rats of a mixed stock, were used as a test system. The cultures were made with reconstituted rat tail collagen. The explants were put on top of the collagen and left to dry for a few hours. A fluid medium consisting of 50 per cent human adult serum, 5 per cent chick embryo extract and 45 per cent Tyrode salt solution increased with glucose to produce a concentration of 500 mg% was then added. Antibiotics were included. Incubation at 37.5°. Growth commenced after 3-4 days and in most cultures a mat of neuroglia cells was found after approx. one week.

Before the tests the cultures were rinsed with Parker 199. The cell suspension to be tested was then added. It was left in contact with the culture for approx. 24 hours and then washed off. Parker 199 was added as a medium. In control cultures glia cells survived for a long time in this medium. Phase contrast photomicrograms were taken before addition of the cells and at regular intervals during the following 48 hours.

## RESULTS

### 1. Scoring of the Tests

"O" Glia cells and mesenchymal parts of the cultures survived well. A strong branching of the glia cell processes was noted.

"+" A slight thinning out of the glia cell mat occurred with de-



Fig 1A

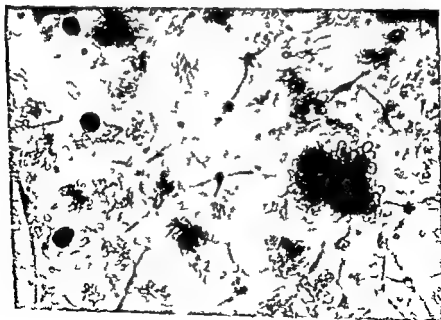


Fig 1B

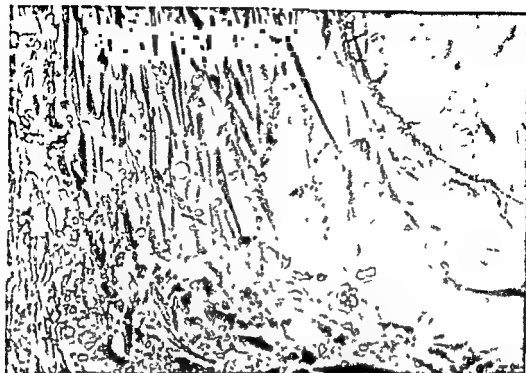


Fig 1C

Fig 1A-C

Phase contrast photomicrogram of a culture of neonatal rat brain. A Culture before addition of sensitized white blood cells. B The same microscopic field 48 hours later. C Another microscopic field showing intact mesenchymal cells.

generation of some glia cells but with good survival of most glia elements, which showed a normal appearance comparable to those in cultures scored "O"

"++" Heavy degeneration of glia cells occurred, leaving mesenchymal elements intact (Fig 1A, B, C). Numerous macrophages could usually be observed. The rapidity of the degeneration varied in different experiments. In most of these, a complete degeneration was found already at 24 hours, but in some not until 48 hours.

Agglutination of lymph cells around glia elements, similar to that described by *Koprowski & Fernandes* (1962), was often seen. In our opinion, however, the glia-cell degeneration is a more positive indication of the effect of the lymph cells.

## 2 Rabbit Experiments

The results are summarized in Table 1.

A) *Non-immunized animals* 10 animals were studied. 6 were scored "O" both 24 and 48 hours after addition of the cells. In the other 4, the cultures were scored "O" at 24 hours and "+" at 48 hours. This faint cytopathic effect can thus occur in normal animals and is therefore without significance.

TABLE 1  
Rabbit Material

Animal at bleed	Days after immunization	Clinical signs of EAE	Duration of clinical signs	Histological signs of EAE	Results of cell test		Note
					After 24 hrs	After 48 hrs	
16	4	0		neg	0	0	
17*	5	0			0	0	
34	7	0		neg	0	0	
51	7	0		neg	0	+	Thoracic cells neg
20	8	0			0	+	Dead in EAE at 10 d
35	8	0		neg	++	++	
40	8	0		pos	0	0	
41*	8	0			++	++	Before immun neg
61	8	0		neg	++	++	Thoracic cells pos
36	9	0		neg	++	++	Before immun neg
52	9	0		neg	0	0	Thoracic cells neg
53	10	0		pos	+	++	
29	10	0		neg	++	++	
37	10	0		neg	0	++	Before immun neg
42	10	0		neg	0	0	Before immun neg
43*	10	0			++	++	Before immun neg
63	10	pos	1	pos	+	++	Thoracic cells pos
44	11	0		neg	+	0	Before immun neg
47§	12	pos	0	pos	++	++	
50	12	0		pos	0	++	
31	13	pos	1	pos	0	++	
41§	13	pos	3	pos	0	0	
21	14	pos	2		0	++	
33	15	pos	2		0	+	
28	19	pos	3	neg	0	0	
49	21	0		neg	++	++	
38	22	pos	1	pos	0	0	Before immun neg
39	26	0		pos	++	++	
64	29	pos	18	pos	0	0	
43§	37	0		neg	0	0	
41	37	pos	4	pos	0	0	

\* First bleeding after immunization

§ Second bleeding after immunization

b) *Animals tested within one week after immunization* 4 animals belong to this group. All were healthy, and the brains studied showed no signs of EAE. In no case was a "++" effect seen.

c) *Animals tested more than one week after immunization* 27 tests were made. Of these, 15 were positive and 12 negative (cf Table 2).

TABLE 2

Summary of Test Results in Animals Bled more than 1 Week after Immunization

	Clinical signs of EAE		Total
	present	absent	
Positive tests	4	11	15
Negative tests	6	6	12



Of the 15 positive tests, only 4 were made on animals showing positive findings of clinical EAE. Of the 11 without clinical signs of EAE, 3 had a positive histology. The 4 animals with clinical signs of EAE had all shown symptoms for less than 2 days. In the tests of three of these animals only a slow degeneration could be observed in the culture. This was not noticeable 24 hours after addition of the cells.

Of the 12 negative tests, 8 animals showed symptoms of EAE and 4 did not. Most of the former 6 had shown the clinical signs for more than 2 days. One of the latter 6 had a positive histology. Another had been positive in an earlier test. The remaining 4 with a negative test in this group may not have been adequately immunized.

As is well known, the timing of the clinical development of EAE is extremely difficult to assess. Nevertheless, the trend of the investigations presented here suggests the possibility of finding a positive test in animals a few days before the appearance of clinical signs of EAE and during the first days of the disease.

Three animals offer some points of special interest because repeated tests were performed on the same material.

*Animal No. 47* This animal was negative 5 days after immunization, positive after 12 days, simultaneously with the development of clinical signs of EAE.

*Animal No. 43* This animal was negative before immunization, positive 10 days after immunization, but never developed clinical signs of EAE. After 37 days the test was again negative, and histology at this stage did not show any signs of EAE. Nevertheless, it is not possible to preclude the presence of a slight EAE.

*Animal No. 41* Cell tests before immunization was negative. At 8 days after immunization, the animal did not show signs of EAE but the cell test was positive. After 10 days pareses developed. On the 13th day the test was again negative, clinical signs and histology then being positive.

In four experiments, the results of blood cell tests were compared with those of thoracic duct lymph cell tests. In two of these cases (Nos. 51 and 52) both blood and lymph cell tests were negative—neither of these animals, examined 7 and 9 days after immunization resp., presented signs of the disease. In the other two cases (Nos. 61 and 62) both blood cell and thoracic lymph cell tests were positive. These animals were examined 8 and 10 days after immunization, the former showing no signs of EAE, the latter exhibiting both clinical and histological signs. A good agreement between white blood cell and lymph cell tests was thus noted.

*D) Animals immunized against pig kidney tissue.* Tests performed after intervals of 9–12 days were all negative.

*E) Rat experiments.* As stated previously by us (1962 a), we had great difficulties in obtaining clinical and even histological signs of EAE in

the rat strain used. In the present material no cases of EAE were manifest. It was possible, however, with thoracic duct lymph cells from injected animals to provoke degeneration phenomena in the glia cultures, comparable to those obtained with rabbit cells. Table II briefly summarizes our results. Lymph from normal rats did not produce this effect.

TABLE 3  
*Rat Material*

Animal number	Days after immunization	Results of cell test	
		after 24 hrs	after 48 hrs
1	9	0	0
40	10	0	0
41	10	0	0
42	10	0	++
49	10	++	++
50	10	0	0
51	12	+	++
52	13	++	++
43	15	0	0
44	15	++	++
45	15	+	++
51	15	++	++
52	15	+	+
46	20	0	++
60	22	0	0
30	23	++	++

#### 4 Killing the Lymph Cells

In a few experiments, rat or rabbit lymph cells, collected through the thoracic duct and found effective in previous tests, were put into the deep-freeze ( $-22^{\circ}\text{C}$ ) for a few days. This treatment killed the cells.

## DISCUSSION

A palant phenomenon has been described in relation to homograft reaction. In the latter experiments, however, the immunologic activity was directed against strain- or species specific antigens—in our experiments against organ-specific antigens. With spinal cord tissue from pigs, the immunizations were made in rabbits and tested on rat cells. The immunologic state of the rabbits can be described as a brain "allergy" resulting in EAE formation. It is not unlikely that the active lymph cells damage glia cells also *in vivo*. It is

Of the 15 positive tests, only 4 were made on animals showing positive findings of clinical EAE. Of the 11 without clinical signs of EAE, 3 had a positive histology. The 4 animals with clinical signs of EAE had all shown symptoms for less than 2 days. In the tests of three of these animals only a slow degeneration could be observed in the culture. This was not noticeable 24 hours after addition of the cells.

Of the 12 negative tests, 6 animals showed symptoms of EAE and 6 did not. Most of the former 6 had shown the clinical signs for more than 2 days. One of the latter 6 had a positive histology. Another had been positive in an earlier test. The remaining 4 with a negative test in this group may not have been adequately immunized.

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*D) Animals immunized against pig kidney tissue* Tests performed after intervals of 9–12 days were all negative.

*3. Rat experiments* As stated previously by us (1962a), we had great difficulties in obtaining clinical and even histological signs of EAE in



generally agreed that the antigen responsible for EAE immunization is made up of myelin or fraction of myelin. Hence, to obtain an effect on glia cells seems to represent the exceptional finding. It should be remembered, however, that the neo-natal glia cells take an active part in myelin-forming processes, and may reasonably be thought to contain myelin antigens. *Olmsted* (1961) has recently shown that not only oligodendroglia cells but also astrocytes take part in myelination in cultures from neo-natal rat brains. We have not yet tried to study the toxic effect of sensitized lymph cells on adult glia tissue.

It should be noted that the lymph cells are able to attack glia cells without the presence of complement. This is in agreement with the findings of, among others, *Goverly* (1960), *Rosenau & Moon* (1961) and *Klein & Sjogren* (1960). The lymph cells must be alive to be effective in the experimental set-up we have used. The lymph cell agglutination, observed by us and by *Koprowski & Fernandes* (1962), may play an important rôle in the cytopathic effect.

Immunized rabbits may show a negative test. In the present material this occurred under the following conditions: 1) Before the end of the first week, all tests performed so far were negative. This was to be expected as a state of hypersensitivity does not arise until at this stage. 2) One animal, which never developed EAE, was negative in the cell test. This is also to be expected. 3) Some animals—approximately half—which developed EAE were also negative. This is at first astonishing. The observations made on animals No. 41 and 43 may help to explain it, as it was apparent in those animals that a positive test may turn negative a few days later. Animal No. 41 showed a positive test just before development of EAE, but already 3 days after the onset of clinical signs of EAE, the test was again negative. A likely hypothesis is that immunologically active cells are withdrawn from the circulation at the development of the disease, possibly by an absorption into the nervous tissue. This idea would agree with the theory suggested by *Waksman* (1959). Animal No. 40 showed no clinical signs of EAE 8 days after immunization, but the histological picture showed an unusually strong cellular infiltration in the brain. This animal had a negative test.

Positive tests were found not only in animals with a manifest EAE. In II tests, positive results were obtained without signs of EAE. 5 of these were made during days 8-10, that is a few days before the expected onset of the disease. This finding agrees with *Stone's* (1961) observation that at EAE transfer experiments between guinea pigs, the best results were obtained a few days before the onset of the disease. *Astrom & Waksman* (1962) obtained successful transfer in rabbits with lymph node cells already before the appearance of clinical signs of EAE. In the sixth case, a positive test was found on the 21st day after immunization but no signs of EAE were present. Our experience, however, is that in a few instances EAE may develop extremely late—up to 6

## INFECTION AND MALIGNANT TUMOURS

### 4 *Comparison between the Effect of Phage Lysates of Haemolytic Streptococci and 'Coley Mixed Toxins' on Brown-Pearce Carcinoma*

By

LEBET ANRFSBURG CHRISTENSEN

Received 14 x 1953

In the latter half of the 19th century several communications appeared on the regression of malignant tumours in connection with acute infections, usually erysipelas (3, 2, 6)

In 1892 W. H. Coley commenced treatment of patients suffering from malignant diseases with a non infectious preparation of haemolytic streptococci and *Bacillus prodigiosus* (*Serratia marcescens*). The reason for including *B. prodigiosus* was the observation that the virulence of the streptococci seemed to increase if they were associated with *B. prodigiosus* in their proliferating stage (18). Preparations of killed haemolytic streptococci alone were only used for a short period, as they appeared to be without any effect. "Coley Mixed Toxins", however, have not acquired importance in the treatment of human tumours, as no tumour-inhibiting effect has ever been convincingly demonstrated in human cancer. A comprehensive survey of the treatment with "Coley Mixed Toxins" was published by Vauts *et al.* in 1953.

Investigating the Shwartzman reaction, Gratia & Linz in 1931 showed that a transplantable guinea pig sarcoma often became necrotic after injection of a filtrate of a culture of *Escherichia coli*, without any sensitizing injection having been given.

During the next few years, this demonstration led to numerous experiments on transplantable tumours in mice, using toxins from various Gram negative bacteria (22).

Shear *et al.* isolated polysaccharides from *E. coli* and *S. marcescens* in 1936 and 1943, respectively. The polysaccharides had the same effect on mouse tumours as the unpurified preparations from Gram-negative bacteria (20, 22). The *S. marcescens* polysaccharide was tested on malignant tumours in human subjects, but no effect could be obtained corresponding to the haemorrhagic necrosis which could be produced in experimental mouse tumours (1, 11, 17, 19).

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mary carcinomas and methylcholanthrene induced tumours in mice and on Rous sarcoma in chickens

In the present investigation, "Coley Mixed Toxins" from the New York Cancer Research Institute were compared with phage lysates of haemolytic streptococci. The comparison was carried out by means of Brown Pearce carcinoma in young rabbits

## MATERIAL AND METHODS

Young male rabbits of a white Danish breed (Copenhagen Whites" from "Hvide sten" the breeding farm of Statens Seruminstitut) were used in the experiments. All animals were between 7 and 9 days old at time of transplantation.

Transplantation to the rabbits was by intraperitoneal injection of a cell sus-

THE FOLLOWING WERE USED IN THE TREATMENT

1) "Coley Mixed Toxins" made available by the New York Cancer Research Institute. The toxins were prepared from *Serratia marcescens* and *Streptococcus pyogenes* and the inhibitory activity towards mouse tumours and the sterility were tested by the New York Cancer Research Institute.

2) Lysates of haemolytic streptococci group A type 12 no 346a (local isolation number). The bacterial culture was lysed by "sewage" phage and filtered sterile through a Berkefeld filter (5). The lysates were prepared by Dr. Ebbe Kjems, Statens Seruminstitut.

3) Human serum broth was used as placebo (13).

All injections were given intraperitoneally. The first injection was on the 2nd day after transplantation; the subsequent injections were given either with constant dosage three times a week for three weeks or with increasing dosage every day for a total of 20 days.

The general condition of the animals was judged on their increase in weight and on the appearance of the

days

The

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on the 20th and 30th day after transplantation

Autopsy of all the rabbits was carried out with the same technique and by the same examiner. On the basis of the autopsy findings the animals were divided into three groups according to the occurrence of metastases (A) and into three groups according to the quantity of tumour tissue (B) (5).

- |   |   |   |
|---|---|---|
| A | 1 | Tumour growth restricted to peritoneum                          |
|   | 2 | Tumour growth in peritoneum and invasion of diaphragm to pleura |
|   | 3 | Metastases at one or more sites                                 |
| B | 1 | No or little tumour tissue                                      |
|   | 2 | Moderate quantity of tumour tissue                              |
|   | 3 | Large quantity of tumour tissue                                 |

## RESULTS

In the *first series of experiments*, the animals were treated according to the scheme which in previous experiments with bacterial lysates had given the best inhibition of the Brown Pearce carcinoma (4). The largest dose which did not affect the general condition of the animals was used for the treatment.

A total of 25 young rabbits were treated with Coley's toxins at con-



Zahl *et al* in 1945 studied 32 different strains of haemolytic streptococci, 25 belonging to group A, 2 to group B, and 5 to group C, using a technique developed on the basis of the effect of Gram-negative bacteria on mouse tumours. They failed to demonstrate any tumour inhibiting effect of the streptococci.

In 1955, Koshimura *et al* reported that the take of Ehrlich ascites carcinoma could be prevented, if the tumour cells were incubated together with living haemolytic streptococci before transplantation. The inhibiting effect could not be demonstrated with heat-killed haemolytic streptococci, nor with living staphylococci, pneumococci or *E. coli*. The same group of investigators demonstrated in 1960 that a cell-free extract of streptococci could also prevent the take of Ehrlich ascites carcinoma.

In 1958, Jordan *et al* demonstrated that a transplantable mouse leukaemia could be inhibited by infection with haemolytic streptococci, if the infection occurred less than 72 hours after transplantation.

In 1959, Ginsburg showed that various proteolytic enzymes which by themselves did not damage the cells of Ehrlich ascites carcinoma, could lyse such cells if the latter had previously been exposed to various streptococcal haemolysins. The effect of streptolysin O was blocked by ascites fluid or normal rabbit serum, whereas these had no effect on streptolysins S and D.

Christensen & Kjems showed in 1959 that a bacteria-free lysate of haemolytic streptococci, produced by lysing the bacteria with streptococcal phage, could inhibit growth of and metastases from Brown Pearce carcinoma in young rabbits.

The above-mentioned experiments with toxins from Gram negative bacteria, together with the old clinical observation of the antagonism between erysipelas and cancer, led Havas *et al* to investigate the tumour-inhibiting effect of a total of 126 preparations of *S. marcescens*, haemolytic streptococci and mixed preparations of the "Coley Mixed Toxins" type. The results were compared with the results of experimental infections with the same bacteria. The experimental technique was based on the experiments mentioned above with mouse tumours which were inhibited by toxins from Gram-negative bacteria. The results were published in 1958 and provided a valuable confirmation of the earlier findings. Both purified and unpurified preparations of *S. marcescens* inhibited Sarcoma 37 in ICR Swiss mice. A mixed preparation of the type "Coley Mixed Toxins" showed the most pronounced effect in proportion to toxicity. Preparations of haemolytic streptococci alone, as well as infections produced by haemolytic streptococci, had no appreciable effect on tumour growth.

In 1961, Havas *et al* reported that mixed preparations could inhibit Sarcoma 37 and Krebs-2 carcinoma in mice, while no effect was found on three other transplantable mouse tumours, on spontaneous mam-

control animals, either in the presence of metastases or in the quantity of tumour tissue (Fig 1 A and 1 B)

A total of 28 young rabbits received treatment with bacterial lysate in constant dosage. In most cases, with the preparations used, the largest dose not affecting the general condition of the animals was 5 ml, but 2.5 ml and 10 ml were also used in some cases. Two of these 28 animals died before the 12th day after transplantation and were therefore excluded from the material. A considerable proportion of the remaining animals showed pronounced inhibition of tumour growth (Fig 1 A and 1 B)

In the second series of experiments the animals received increasing doses, this being the system of dosage used by W. B. Coley and now recommended by the New York Cancer Research Institute (16)

The rapid growth and dissemination of the Brown Pearce carcinoma in the young rabbits necessitated a rapid increase in dosage

Treatment with "Coley Mixed Toxins" was instituted with 0.05 ml, the dose increasing to 1.0 ml in the course of 20 days. Treatment with bacterial lysate was introduced with 0.75 ml and terminated with 15 ml

A total of 18 animals were treated with "Coley Mixed Toxins" in increasing doses. All of them survived long enough to provide comparison with the control animals. The treated animals did not differ from the control animals as to presence of metastases and quantity of tumour tissue (Fig 1 A and 1 B)

TABLE 1

	No of rabbits at commencement of experiment	No of rabbits dead before 12th day after transplantation	No of rabbits with adequate observation period	No of rabbits with out vital tumour tissue at autopsy
Coley Mixed Toxins constant dose	25	5	20	0
Untreated	45	0	45	0
Bacterial lysate constant dose	28	2	26	8
Placebo treated	28	1	27	0
Total	126	8	118	8

TABLE 2

	No of rabbits at commencement of experiment	No of rabbits dead before 12th day after transplantation	No of rabbits with adequate observation period	No of rabbits with out vital tumour tissue at autopsy
Coley Mixed Toxins increasing dose	18	0	18	0
Untreated	22	2	20	1
Bacterial lysate increasing dose	18	1	17	0
Placebo treated	18	1	17	0
Total	76	4	72	1

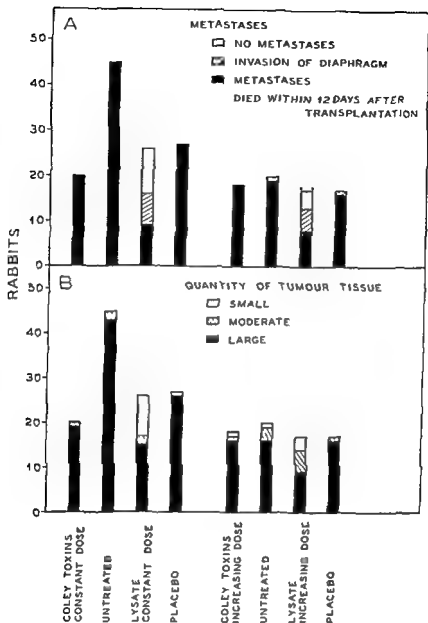


Fig 1 A and B

Metastases and quantity of tumour tissue at autopsy

stant dosage Twelve of these animals received 0.25 ml and 13 received 0.50 ml per injection

Five of the 25 animals died before the 12th day after transplantation *i.e.* before the tumour had developed sufficiently Four of these five animals had received 0.5 ml and the fifth 0.25 ml per injection 0.5 ml three times a week thus turned out to be too much, although preliminary experiments on young rabbits without tumours had shown that the animals tolerated this dose

The remaining 20 animals did not differ from the corresponding



A total of 18 animals received treatment with bacterial lysate in increasing doses. One of the animals died before the 12th day after transplantation and was therefore excluded from the material. There was inhibition of the tumour growth in a number of the remaining animals. Nine of the 17 animals showed no metastases, but in five of these 9 animals the diaphragm had been invaded by tumour tissue, i.e. the animals showed *incipient dissemination of the tumour* (Fig. 1 A and 1 B).

The result of treatment with increasing doses of bacterial lysate was poorer than that obtained in the first series using a constant dosage, even though the total dosage was slightly larger in the second series. The difference is clearly demonstrated on comparing the findings of vital tumour tissue at autopsy. After treatment with constant dosage, 8 of the 26 lysate-treated animals were free from vital tumour tissue. After treatment with increasing dosage, all the 17 lysate-treated animals showed vital tumour tissue at autopsy (Table 1 and 2).

## DISCUSSION

In the experiments reported here, "Coley Mixed Toxins" had no effect on Brown-Pearce carcinoma under conditions where lysates of haemolytic streptococci had an inhibitory effect on the tumour.

Using a technique suitable for demonstrating inhibition of mouse tumour by toxins from Gram-negative bacteria, other workers have been unable to demonstrate tumour-inhibiting effects of haemolytic streptococci, which constitute the one component of "Coley Mixed Toxins" (10, 24).

No inhibition of Brown-Pearce carcinoma could be found with Shear's polysaccharide, produced from *Serratia marcescens*, the other component of "Coley Mixed Toxins" (23). On the other hand, Shear's polysaccharide, like "Coley Mixed Toxins", is effective against a number of mouse tumours (20, 21, 22, 1, 10, 9).

If the results obtained in the above mentioned experiments are compared, the conclusion must be that the effect of "Coley Mixed Toxins" in animal experiments is due to toxins from *Serratia marcescens*. The experiments do not exclude the possibility that "Coley Mixed Toxins" contain active substances from haemolytic streptococci, but such substances have shown no appreciable effect under the experimental conditions so far employed, excepting a possible slight increase in the inhibition of Sarcoma 37 (10).

## SUMMARY

A comparison is made between the effect of "Coley Mixed Toxins" and the effect of phage-lysed haemolytic streptococci, in young rabbits with Brown-Pearce carcinoma. "Coley Mixed Toxins" cannot be shown to have any *inhibiting effect* on the tumour, under experimental con-

## A NEW FERMENTATION MEDIUM FOR N. GONORRHOEAE, HAP-MEDIUM. INFLUENCE OF DIFFERENT CONSTITUENTS ON GROWTH AND INDICATOR COLOUR

By

INGMAR JUELIN

Received 14 VII 62

A bacteriological diagnosis of *N. gonorrhoeae* cannot be based solely on a demonstration of Gram negative diplococci presenting a characteristic colonial appearance and giving a positive peroxidase reaction while incapable of growth on ordinary agar at 22° C. A decisive diagnostic criterion must be whether the bacterium is able to ferment dextrose but not maltose, laevulose, or sucrose. In view of the medicosocial consequences of a positive diagnosis the evidence on which such a diagnosis is based must be absolutely certain and conclusive.

The special growth stimulating factors required by *N. gonorrhoeae* (1, 6), however, have complicated the elaboration of a fermentation medium providing growth in combination with a distinct colour change. Several media have been composed which may be used for most *N. gonorrhoeae* strains (1, 3, 10, 16, 18) but they are not always reliable or poor growth is obtained.

In recent years the incidence of *N. gonorrhoeae* has increased considerably, mainly owing to the treatment of gonorrhoea with penicillin and other antibiotics (15, 16, 20). In 1958 Reyn *et al.* (14) could report a markedly increased frequency, amounting to 40 per cent non-fermenting strains.

Many fermentation media are of a semi-fluid consistence (3, 11, 18), and while contained in test-tubes require heavy inoculation of gonococci. It is possible only with difficulty to decide whether the inoculate is contaminated or not. Nor can the degree of growth be conclusively estimated and related to the degree of dextrose fermentation. Up to now, also solid fermentation media have required heavy inoculation for distinct indicator colours to appear (1, 10, 16) and naturally to establish the purity of a culture under such conditions must involve difficulties.

When it was found that certain strains were incapable of fermenting

- 21 *Shear M J & Perrault A* Chemical treatment of tumors IX Reactions of mice with primary subcutaneous tumors to injection of a hemorrhage producing bacterial polysaccharide *J Nat Cancer Inst* 4 461 1944
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- 23 *Westfall B B & Dunn T B* Blood uric acid and proteose body temperature and glomerular clearance of rabbits implanted with Brown Pearce carcinoma and treated with polysaccharide from *Serratia marcescens* *J Nat Cancer Inst* 7 115 1946
- 24 *Zahl P A Starr M P & Hutner S H* Effect of bacterial toxins on tumors VII Tumor hemorrhagic factor in bacteria *Am J Hyg* 41 41 1945

## MATERIALS AND METHODS

### Test Strains

The test strains used for preliminary investigation included different strains of *N. gonorrhoeae*, *N. meningitidis*, and *N. catarrhalis* isolated from routine diagnostic material. The final medium was tested with selected atypical strains of *N. gonorrhoeae* and in addition to these with 253 consecutively isolated strains.

### Inoculation and Incubation

Broth suspensions of pure bacterial cultures were employed as inoculates. A platinum loop was charged with the suspension and streaked across a quarter of each medium. The plates were incubated at 37° C for 18-22 hours in covered, circular plastic jars in the presence of 8-10 per cent CO<sub>2</sub>. As investigations proceeded the importance of sufficiently moist air became increasingly evident, and a water-soaked foam plastic pad was added to each jar (a jar of two litres was calculated to require a pad of 2 × 5 × 5 cm).

### Media

All the time while the experiments were being carried out each new medium composition was compared to both "chocolate ascitic agar" and "chocolate ascitic fermentation medium". In addition to these, the fermentation medium in use at the Statens Seruminstitut, Copenhagen referred to below as "fermentation medium CPH", was sometimes employed.

### Chocolate Ascitic Agar

Broth agar base: 1.4 per cent Japan shred agar in human placenta infusion (500 g human placenta and 100 ml aq. dest. see HAP medium) containing 1 per cent peptone (Orthana special), 0.2 per cent Na<sub>2</sub>HPO<sub>4</sub> × 7 H<sub>2</sub>O and 0.2 per cent NaCl.

100 ml of the plasma has been replaced with placenta broth and added and the mixture is kept at 80° C for 20 minutes and frequently shaken. It is cooled down to 55° C and mixed with 150 ml of sterile ascitic fluid preheated to 52° C. The medium is immediately poured into plastic Petri dishes to form a 4-5 mm layer.

### Chocolate Ascitic Fermentation Medium

Preparation: 1.4 per cent Japan shred agar in human placenta infusion (500 g human placenta and 100 ml aq. dest. see HAP medium) containing 1 per cent peptone (Orthana special), 0.2 per cent Na<sub>2</sub>HPO<sub>4</sub> × 7 H<sub>2</sub>O and 0.2 per cent NaCl. 1 add a little rose, 1 for

### Fermentation Medium CPH

1.4 per cent Japan shred agar in human placenta infusion (500 g human placenta and 100 ml aq. dest. see HAP medium) containing 1 per cent peptone (Orthana special), 0.2 per cent Na<sub>2</sub>HPO<sub>4</sub> × 7 H<sub>2</sub>O and 0.2 per cent NaCl. 1 per

### Evaluation of Medium Compositions

Each medium was tested with 253 consecutively isolated strains of *N. gonorrhoeae*, *N. meningitidis*, and *N. catarrhalis*. The final medium was tested with selected atypical strains of *N. gonorrhoeae* and in addition to these with 253 consecutively isolated strains.



dextrose on ordinary media although growth was good and inoculation heavy investigations were initiated in 1957 to obtain better media if possible

A good fermentation medium should.

- 1 yield good growth of all normal *N. gonorrhoeae* strains even with a moderate or sparing inoculation
- 2 give a distinct, easily seen but sharply defined colour change in the medium
- 3 provide sufficient growth to ensure a distinct colour change with, preferably, all atypical strains
- 4 make it possible to observe contaminating bacterial colonies
- 5 be easily prepared without different qualities in different batches

### *Theoretical Motivations for the Choice of Test Substances*

The choice of the tested ingredients is partly based on instructions obtained from manuals as regards the various substances generally used and the approximate effect to be expected from these

The importance of choosing a special basic broth was studied, at first partly for economical reasons, placenta infusion broth being compared with other broths

Since certain amino acids have been found to inhibit the growth of *N. gonorrhoeae* (5, 18), a number of peptones of different qualities and partially known structures were chosen

Since agar also may contain inhibitory substances (7), two types were utilized first, ordinary Japanese shred agar, the quality of which may vary considerably from time to time, and next, Bactoagar, pure and standardized

The medium should be of a fairly solid consistence so that it may be easily inoculated on the surface but at the same time it is exceedingly important that the medium readily provides sufficient moisture in order to stimulate bacterial growth. The addition of alginate seemed a conceivable answer to this problem

The addition of heated blood results in an opacity of the medium and since it is of a strong brown colour in itself the observations of any other colour will be impaired. The problem was therefore to find a transparent solution which did not affect the colour of the medium itself but could be used as a substitute for hematinized blood

Buffering a fermentation medium is not usually recommended but if the intensity of bacterial growth is great enough the medium must be buffered in such a way that the colour indications while being perfectly distinct are limited to a comparatively small area near the pH changing bacterial growth

TABLE I  
Combinations of Various Ingredients Tested

Ingredients	Amounts tested	Combinations	UAP ↓
Tryptophan (P, pc)	1100 ml	■■■■■■■	
G C Medium Base Difco	36 g	■■■■■■■	
Pectone (CI Al)	20 g	■■■■■■■	
Neopeptone Difco	20 g	■■■■■■■	
Bact peptone	20 g	■■■■■■■	
Bactotrypticase	20 g	■■■■■■■	
Bacto Beef extract	0.304 g	■■■■■■■	
Placenta human	500 g	■■■■■■■	
Irresol Pectone N, J	101420 g	■■■■■■■	
Difco		■■■■■■■	
Dilum hydregen	0.2024025 g	■■■■■■■	
Thiosulfate x 210	0.703605 g	■■■■■■■	
Sodium chloride	ad 100 ml	■■■■■■■	
Aqua destillata	0.0400480075 g	■■■■■■■	
Benlred Merck	0.2101430 g	■■■■■■■	
Dextrose		■■■■■■■	
Agar agar (Shrelingar Japan)	16 g	■■■■■■■	
Bacto Agar	1213151820 g	■■■■■■■	
Haemin (b vine) crystalized Sigma	0.000100010004001 g	■■■■■■■	
Sodium alginate	0.12 g	■■■■■■■	
Aceite fluid	250300360 ml	■■■■■■■	
Serum inactivated	100 ml	■■■■■■■	
Haemin (human) McQuarrie & Benlams	50100150 ml	■■■■■■■	
Bacto Supplement B	103050 ml	■■■■■■■	
Bacto Yeast Extract	0.5 g	■■■■■■■	

The intensity of indicator colour change was judged by two criteria: first the degree of contrast when compared to the colour of the medium itself; second by the area of colour indication in relation to that of bacterial growth. 4 = a distinct yellow colour change of the whole area of growth and a surrounding zone of 2-4 mm; 3 = a distinct yellow colour in the whole area of growth; 2 = a distinct yellow colour involving about 2/3 of the whole area of growth; 1 = a yellow colour only in the middle of the incubated area or at the site where the richest growth is found; 0 = no visible colour change.

### *Examination of Different Medium Compositions The Construction and Testing of Medium Varieties*

Different combinations and amounts of ingredients were studied in more than 100 medium varieties. For practical reasons a complete examination of every mathematically conceivable combination has not been made.

In Table 1 most of the examined varieties are represented schematically. Among the combinations examined the one described below proved to be the best as regards growth intensity of both normal and atypical strains of *N. gonorrhoeae*, and also as regards distinctly different colour changes, the sizes of single colonies, and the degree of solidity as related to the moisture of the medium.

#### *HAP Medium*

**Placenta infusion**—fresh human placentas are ground after removal of membranes and umbilical cords. The amount thus obtained is weighed and mixed with twice as much aqua dest. and kept at 4°C during the night. On the next day the mixture is boiled for 20 minutes and paper-filtered, yielding a clear fluid which is dispensed into 1-litre Erlenmeyer flasks and autoclaved at 120°C for 20 minutes.

#### *Broth agar base*

Placenta infusion	200 ml
Sodium chloride 7 per cent solution in placenta infusion	60 ml
Disodiumhydrogen phosphate $\times 2 \text{ H}_2\text{O}$ 2 per cent solution in placenta infusion	60 ml
Proteose peptone No. 3 (Difco) 10 per cent solution in placenta infusion	60 ml
Sodium alginate (see to RPC 1959)	0.6 g
Bacto agar (Difco)	6.0 g

These ingredients are mixed in each of four flasks and are then autoclaved at 120°C for 20 minutes. The broth agar base is cooled to 55°C.

#### *HAP-Medium*

Broth agar base	380 ml
Ascorbic fluid preheated to 52°C	180 ml
Phenol red (Merck) 0.4 per cent water solution	6 ml
Hemin (Sigma) 0.2 per cent water solution	10 ml
Sugar 20 per cent solution in placenta infusion	30 ml

The sterile ingredients are mixed at 52-55°C and pH is adjusted to 7.7 with 5% sodium hydroxide. The medium is poured into plastic Petri dishes to make a 4 mm layer.

**Ascorbic fluid**—each fresh batch is sterilized by filtration (Seitz filter Fk52) and compared to earlier batches as regards growth-stimulating effect on strains of *N. gonorrhoeae*, *Streptococcus pyogenes*, *Haemophilus influenzae* and *Diplococcus pneumoniae*.

**Phenol red**—Merck 0.4 per cent solution in aqua dest. is autoclaved at 120°C for 20 minutes.

**Hemin** bovine, 2 x crystallized, Sigma (in Sweden represented by Chemilia) 0.2 per cent solution in aqua dest 0.15 g hemin crystals are dispersed in 75 ml of aqua dest after which 1 a ml 3% sodium hydroxide are added. Shake well to dissolve crystals in order to obtain a perfectly clear dark green solution. This solution is then sterilized by filtration (Seitz filter Ek52).

**Sugar** 20 per cent solution of dextrose, maltose, sucrose or laevulose in placenta infusion. The solutions are sterilized by filtration (Seitz filter Ek52).

### *Discussion of the Ingredients of the Medium*

Some observations of the importance of the various constituents deserve to be mentioned.

The ability of the medium to stimulate growth is markedly influenced by different infusion materials. Horse meat infusion (not included in Table 1) as well as beef extract broth or tryptic digest of horse meat (12) were much inferior to human placenta infusion as regards number and size of colonies. At the Statens Seruminstitut, Copenhagen (15), it was found, more or less at the same time, that ox heart infusion while considerably better than ox meat infusion compared unfavourably with human placenta infusion. The atypical strains especially grow much better if the infusion is based on human placenta.

Comparisons of the various peptones proved Proteose peptone No 3 to be entirely superior to the rest (Neopeptone, Bactotryptose, Bacto peptone, and Peptone UCLAF). According to an analytical scheme in the Difco manual, Proteose peptone contains, among other things, relatively more primary and secondary proteose N, diamino N, and cystine than the others but less ammonia N, peptone N, and free amino N. A possible explanation of the superiority of Proteose peptone may consequently be its low content of free amino N or the proportions of its amino acids.

Ascitic fluid possesses the best growth-stimulating power and cannot be replaced by Bacto supplement B, Bacto-yeast extract, or a combination of these two. Attempts to replace ascitic fluid by other substances are being carried out but have not yet been terminated. (Horse serum can not be substituted for ascitic fluid in fermentation media).

There need be no doubt, however, that, next to ascitic fluid, hemin is the most active growth stimulating factor. The first hemin solution to be utilized was prepared according to a modification of the method described by McQuarrie & Benjamins (8), and this hemin solution was standardized colorimetrically. The fact that this procedure was both lengthy and laborious, however, led to the adoption of crystallized hemin which meant a great simplification. The importance of the thermally stable A-factor in heated blood has been known for a long time but, apparently, crystallized hemin has not previously been employed for this kind of medium. Neither Bacto-supplement B nor Bacto-yeast extract, whether alone or in combination, are able to reproduce the effects of this hemin solution.

The type and concentration of indicator may effect growth. Litmus, bromthymol blue, and neutral red proved unsuitable for several rea-

sons, whereas even a very low concentration of phenol-red sufficiently coloured the medium and gave a clearly visible reaction when the sugar was fermented. Attempts to obtain still sharper contrasts by increasing the concentration of phenol-red were unsuccessful, however, owing to depression of gonococcal growth.

The profuse growth developing on the medium necessitated the addition of some buffering substance to prevent a too wide-spread colour reaction when a sugar is being fermented. The plastic Petri dishes applied had diameters of 9 cm and the amount of buffer was adjusted in such a manner as to make room for 4-6 triangleshaped streaks of gonococci. In spite of a distinct yellow colour change under and immediately surrounding the bacterial growth, a zone of unaffected medium should persist between the coloured areas.

### EXPERIMENTAL—RESULTS—DISCUSSION

Extensive preliminary experiments provided information about such factors as would contribute to the best fermentation medium. These results led to the elaboration of a new test series for the final tests which were performed on the last 30 or thereabout of the medium varieties.

Ten gonococcal strains showing varying susceptibility to penicillin and streptomycin were selected, suspended in placenta infusion broth and standardized colorimetrically. These suspensions were then diluted with placenta infusion broth in the following proportions:  $1/5$ ,  $1/50$ ,  $1/250$ ,  $1/500$ ,  $1/1250$  and  $1/2500$ . One loopful from each of the six dilutions of the ten gonococcal strains was used to inoculate one third of each plate. The medium varieties were examined in groups of six to eight and compared with 'chocolate ascitic agar' and 'chocolate ascitic fermentation medium' (4 total of 480-640 inoculations in each of the experimental series).

After incubation at  $37^{\circ}\text{C}$  for 20 hours in the presence of  $\text{CO}_2$  the intensity of growth and induction colour with each dilution were estimated and graduated separately from

calculated for media were co of all ten strains

best medium composition of the series in question

Tables 2 and 3 exemplify the results obtained from one of these test series where the differences between various media depended on the presence or absence of disodiumhydrogen phosphate combined with different kinds and concentrations of hemin.

Table 2 gives the growth and colour change of the  $1/5$  diluted bacterial suspensions (only 9 strains are represented since one was contaminated). The table is useful in deciding whether any medium composition is wholly unsuitable for a strain or is less able than the rest to produce satisfactory colour in spite of good growth. In Table 3 the points gained by each of the six dilutions with each strain and each medium composition have been added up. The total sum thus obtained indicated that the hemin solution prepared in the laboratory is less favourable to growth

TABLE III  
*Examples of Evaluation and Registration of Density of Growth and Intensity of Indicator Colour on Medium Varieties*

Hemin, McQ & B Hemin Sigma Na <sub>2</sub> HPO <sub>4</sub> × 2 H <sub>2</sub> O N gonorrhoeae strain	Composition of medium											
	5.0 per cent			5.0 per cent			0.0001 per cent			0.001 per cent		
	0.2 per cent			0.2 per cent			0.2 per cent			0.2 per cent		
	colour	growth		colour	growth		colour	growth		colour	growth	
R	1	9		2	9		3	9		5	12	
S	6	5		0	2		1	3		0	2	
T	6	13		8	13		8	14		5	12	
U	1	7		1	7		4	9		5	11	
V	4	6		4	7		3	6		5	13	
W	0	2		0	3		0	3		2	5	
X	2	4		0	2		0	3		1	4	
Y	3	4		2	3		3	4		0	4	
Z	0	3		0	3		1	4		3	7	
Total sum	19	53		17	49		23	55		23	65	
Sum of colour and growth	72			66			76			88		

Inocula were taken from a dilution series (1/5, 1/50, 1/500, 1/1250, 1/2500) of a dense suspension of *N. gonorrhoeae* in placenta-infusion broth. One loopful was spread over 16 of each plate. Growth and colour were classified from 0-4. Figures indicate the sum of growth or colour "points" from the six dilutions.







than the one based on the crystallized "Sigma" hemin. The stronger of two hemin concentrations yielded the best result. Phosphate is beneficial to growth to some extent but at the same time it exercises a certain inhibitory effect on the colour change in such media as are characterized by the most luxuriant growth.

Taken separately the differences may appear small indeed, owing to the close resemblance in composition between these medium varieties and HAP-medium, but the results indicate the principles on which to base the ideal medium.

The last medium compositions were also studied by counting the number of colonies from a standardized inoculation.

Pure cultures of 8 selected gonococcal strains were used to charge a little less than half a loopful with bacteria which were then suspended in 5 ml of placenta infusion broth. Two dilutions were made of this broth 1/50 and 1/2500. By means of a 0.25 ml pipette 0.2 ml of each dilution were transferred to Petri dishes containing different medium varieties and the inoculate was then distributed over the whole area using a bent glass rod. After incubation for 20 hours at 37° C in the presence of CO<sub>2</sub> the colonies were counted. Four of the strains (J-M) numbered 200 colonies at the most, the other four (N-Q) 400 colonies at the most (figures have been rounded off to nearest ten; see table).

The results have been summarized in Table 4. The addition of alginate would not seem to affect the growth in this investigation. An increased amount of hemin means a certain increase in the number of colonies, too. In most cases this action is insignificant but with one of the strains (strain Q), a threefold increase of the amount of hemin has a notably stimulating effect on both dilution 1/50 (from 30 colonies to almost confluent growth) and dilution 1/2500 (from 7 to more than 400 colonies). The advantages of the new HAP-medium is best seen in connection with gonococcal strains of this very type.

Only small differences can be detected from a comparison between

*Density of Growth of eight Selected Strains of *Neisseria gonorrhoeae* on Variants of HAP medium*

Variants of HAP medium			<i>Neisseria gonorrhoeae</i> strain					
$\text{Na}_2\text{HPO}_4$ per cent	Alginate per cent	Hæmin per cent	J	K	L	M	N	O
			10	2500	50	10	2500	50
1 %		0.001	confl	confl	confl	>200		
2 %	0.1	0.001	contaminated		confl	contam	confl	confl
3 %	0.2	0.001	confl	>200	confl	confl	confl	confl
4 %	0.2	0.002	confl	>200	confl	50	confl	confl
5 %	0.2	0.003	confl	>200	confl	>200	confl	confl
Ferment medium CPH			confl	>200	0	10	>200	confl
Chocolate ascitic agar			confl	confl	contam	>200	confl	confl

Inocula prepared from suspensions of ten colonies in five ml of placenta infusion broth. 400 at most for strains N-Q. For convenience the values obtained have

TABLE 5

*Density of Growth and Intensity of Indicator Colour of 253 Consecutive Isolated Strains of V. gonorrhoeae on HAP Medium Fermentation Medium CPH and Chocolate-Acetic Fermentation Medium*

Degree of growth and colour intensity	HAP medium					Ferment medium CPH			Chocolate acetic ferment medium			
	Dextrose colour	Dextrose growth	1 acetal growth	Malt growth	Sacch growth	Dextrose colour	Dextrose growth	Malt growth	Dextrose colour	Dextrose growth	1 acetal growth	Malt growth
4 Number Percent	187 73.9	199 78.7	201 79.4	204 80.6	203 80.2	11 4.3	67 26.5	88 22.9	199 78.7	204 80.6	211 83.4	209 82.6
3 Number Percent	27 10.6	34 13.4	40 15.8	38 15.0	34 13.4	33 13.1	10 7.5	25 9.9	35 13.8	36 14.2	32 12.6	34 13.4
2 Number Percent	24 9.5	16 6.3	8 3.2	5 2.0	12 4.8	34 13.4	21 8.3	15 5.9	9 3.6	9 3.6	7 2.8	9 3.6
1 Number Percent	10 4.0	3 1.2	1 0.4	6 2.4	2 0.8	27 10.7	39 15.4	41 16.2	50 19.8	3 1.2	2 0.8	1 0.4
0 Number Percent	5 2.0	1 0.4	3 1.2	0 0	2 0.8	148 58.5	107 42.3	114 45.1	24 9.5	8 3.2	1 0.4	0 0

The inoculum one loopful of a suspension of ten colonies in 10 ml broth, was spread over a quarter of each plate



Fig 1

Three strains of *N gonorrhoeae* on HAP medium one normal (top) and two atypical poorly growing strains (left and right) Note the different size of the colonies (Incubated at 37° C for 48 hours in the presence of CO<sub>2</sub>)



Fig 2

Strains of *N gonorrhoeae* (right), *N succat* (top) and *N meningitidis* (left) on HAI medium with dextrose (D) lactulose (L) maltose (M) and sucrose (S) (Incubated at 37° C for 18 hours in the presence of CO<sub>2</sub>)

TABLE 6

*A Detailed Report of such N. gonorrhoeae Strains as Showed none or merely Weak Dextrose Fermentation on Chocolate Asetic Fermentation Medium or HAP Medium when Primarily Diagnosed, Followed by the Results of a Second Cultivation of some of the Strains*

	Results on primary isolation					Results on control														
	Colour classified as					Growth classified as					Colour classified as					Growth classified as				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Chocolate asetic fermentation medium	24	-	-	-	-	7	2	4	3	8	6	1	4	-	-	8	-	-	-	8
HAP medium	3	5	5	1	11	1	-	5	2	16	-	-	3	-	8	-	-	-	-	11
Chocolate asetic fermentation medium	50	-	-	-	-	5	17	28	1	1	8	-	-	-	-	2	8	-	-	-
HAP medium	3	4	10	9	24	-	2	6	14	28	-	1	1	1	7	-	-	-	-	3
HAP medium	5	-	-	-	-	1	1	-	1	-	1	-	1	-	2	-	-	1	1	8
Chocolate asetic fermentation medium	2	3	-	-	-	1	-	-	3	1	2	-	2	-	-	1	-	1	-	2
HAP medium	70	-	-	-	-	2	7	1	-	-	-	-	1	-	3	-	-	-	-	2
Chocolate-asetic fermentation medium	5	4	1	-	-	2	-	5	2	1	1	-	1	-	-	1	-	-	-	1

growth has been considered maximal and with an additional 13 per cent nearly maximal. On "fermentation medium CPH", however, only 25 per cent attained maximal growth, and a further 8 per cent nearly maximal growth, while no less than 42 per cent of the gonococcal strains are unable of any growth whatsoever with ordinary inoculation.

The most important distinctions between the different media though are found when we compare the depth of their colour indication. The colour given 2 points on HAP-medium is much more easily discernible than the correspondent 2-point colour on "chocolate ascitic fermentation medium" owing to the transparency and better contrast effects of HAP medium. To obtain a proper comparison it is thus necessary to take points 2, 3, and 4 on HAP-medium together. It then appears that 95 per cent of the strains show a very clear colour change on HAP medium as compared with 70 per cent on "chocolate ascitic fermentation medium", and merely 30 per cent on "fermentation medium CPH". The colour changes given 1 point only constitute 4 per cent on HAP medium, and approximately 20 per cent on "chocolate ascitic fermentation medium", but here, too, it must be borne in mind that the 1-point colour is much better seen on HAP medium than on "chocolate ascitic fermentation medium".

These gonococcal strains which failed to present a colour change in this routine investigation amount to 2 per cent on HAP medium, 10 per cent on "chocolate ascitic fermentation medium", and 59 per cent on "fermentation medium CPH".

Even for routine diagnostic purposes the new HAP-medium has thus shown its superior qualities in spite of the fact that the density of the seeding suspension has been chosen explicitly so as to give the ideal density of colonies for purposes of resistance testing (by a diffusion method using antibiotic discs of paper (2), on "chocolate ascitic agar"). "Ideal density of colonies" in this case implies closely arranged, nearly confluent colonies giving a strong impression of a granular surface.

Some of the gonococcal strains of the routine investigation reported above, which did not grow or gave a 1-point-colour only, have been submitted to further study to see whether the medium is unsuitable for these strains or whether the unsatisfactory results were purely accidental.

Unfortunately, not all of these strains have been accessible to observation. Table 6 presents the results of the primary cultivation as well as the results of a secondary investigation of the strains at disposal. In 24 of the gonococcal strains which showed no colour change on "chocolate ascitic fermentation medium", the reason in seven cases was absence of growth but on HAP-medium all except one grew, and all except two gave a colour change. Out of these 24 strains, eleven were inoculated a second time but even then 3 were incapable of growth on "chocolate ascitic fermentation medium" and three others gave no colour change although growth was satisfactory. But on HAP-medium

give good growth and indication colour on HAP medium whereas one did not grow on 'chocolate asetic fermentation medium'

The conclusion must be that out of 253 investigated strains, one strain gave no colour change on HAP-medium after 20 hours of incubation and one strain, inaccessible to subsequent re-examination, did not grow

Since one of the control media was 'fermentation medium CPH' which was in use at the Statens Seruminstitut, Copenhagen, Dr A Revn who is director of the gonococcal department there did kindly subject the new HAP medium to further examination. Eight specially selected gonococcal strains, consisting of 2 normal and 6 atypical, poorly growing strains were employed, and two dilutions, 1/1 and 1/10, were prepared of each strain. The undiluted suspension 1/1, contained  $10^8$   $10^9$  bacteria per ml. The plates were incubated as usual, colonies were counted, and their size and intensity of colour estimated.

The results are presented in Table 7, showing the numbers of colonies on HAP medium, HAP medium without hemin and 'fermentation medium CPH'. All of the strains exhibit excellent growth on HAP medium, averaging approximately 500-600 colonies with dilution 1/1. Removal of

TABLE 8

Results of investigation of *N. gonorrhoeae*

Gonorrhoeae strain	Dilut	HAP medium		HAP medium without hemin		Ferment medium CPH	
		Colony diameter		Colony diameter		Colony diameter	
		smallest mm	largest mm	smallest mm	largest mm	smallest mm	largest mm
A	1/1	1.0	2.0	0.5	1.0	-	-
	1/10	1.0	2.0	0.5	1.0	-	0.25
B	1/1	0.5	2.0	0.5	1.5	-	1.0
	1/10	1.5	2.5	0.5	1.0	-	0.5
C	1/1	0.25	1.0	0.5	0.5	-	-
	1/10	0.25	1.0	-	0.5	-	-
D	1/1	0.5	1.0	-	1.0	-	-
	1/10	0.5	1.0	-	-	-	-
E	1/1	0.5	1.5	-	0.5	-	-
	1/10	0.5	2.0	-	-	-	-
F	1/1	0.5	2.5	0.5	2.0	-	-
	1/10	0.5	2.0	0.5	1.0	-	-
G	1/1	0.25	0.25	0.5	0.5	-	-
	1/10	0.25	0.25	0.5	0.5	-	-
H	1/1	0.25	0.25	0.25	0.5	-	-
	1/10	0.25	0.25	0.25	0.25	-	-
Average value	1/1	0.5	1.3	0.4	0.9	-	(0.1)
	1/10	0.6	1.4	0.5	0.7	-	(0.1)

all of the strains grew fermenting dextrose with a resulting colour change

All of the 50 strains which gained 1 point for colour on "chocolate ascitic fermentation medium" grew satisfactorily, and on HAP-medium 43 produced a distinct colour change, 4 strains a weak one, and 3 strains no colour change. Ten of these strains have been controlled and found to give good growth but, in one case, no indication colour developed on "chocolate ascitic fermentation medium"

As stated above, five strains developed no colour change on HAP-medium on routine diagnosis. Out of these, two strains grew poorly or not at all, while "chocolate ascitic fermentation medium" gave no growth in one case and no colour change in another. Four of these strains were re-examined with the result that three showed good growth and colour on HAP-medium whereas the fourth merely developed moderate growth and no colour after 20 hours of incubation. On "chocolate ascitic fermentation medium" two strains were still incapable of colour change, one simply because it did not grow.

Ten strains had gained 1 point for colour on HAP-medium, none of them growing entirely satisfactorily. On "chocolate ascitic fermentation medium" 5 of these gave no colour change, in two cases because of absence of growth. Four of these strains were re-examined and found to

TABLE 7

*Results Obtained from Counting the Number of Colonies on HAP Medium, HAP Medium without Hemin, and Fermentation Medium CPH, as Carried out at Statens Serum Institut Copenhagen on two 'Normal' Strains (A-H) and six Selected Atypical Strains (C-H) of N. gonorrhoeae*

N. gonorrhoeae strain	Dilut	HAP medium		HAP medium without hemin		Ferment medium CPH	
		Dextrose	Maltose	Dextrose	Maltose	Dextrose	Maltose
A	1/1	283	414	171	387	0	0
	1/10	38	48	26	31	0	1
B	1/1	680	720	107	27	0	3
	1/10	76	79	5	8	2	0
C	1/1	125	145	5	16	0	0
	1/10	9	21	0	1	0	0
D	1/1	458	590	0	1	0	0
	1/10	42	77	0	0	0	0
E	1/1	526	717	1	8	0	0
	1/10	59	72	0	8	0	0
F	1/1	354	459	20	118	0	0
	1/10	38	52	4	3	0	0
G	1/1	1486	1526	1474	1730	0	0
	1/10	147	182	89	190	0	0
H	1/1	452	540	374	590	0	0
	1/10	32	52	24	43	0	0
Average number	1/1	546	639	269	378	0	0.4
	1/10	55	73	19	34	0.3	0.1

give good growth and indication colour on HAP medium whereas one did not grow on "chocolate ascitic fermentation medium"

The conclusion must be that out of 253 investigated strains, one strain gave no colour change on HAP medium after 20 hours of incubation and one strain, inaccessible to subsequent re-examination, did not grow

Since one of the control media was "fermentation medium CPH" which was in use at the Statens Serum Institut, Copenhagen, Dr A. Reyn, who is director of the gonococcal department there did kindly subject the new HAP-medium to further examination. Eight specially selected gonococcal strains consisting of 2 normal and 6 atypical, poorly growing strains were employed, and two dilutions, 1/1 and 1/10, were prepared of each strain. The undiluted suspension, 1/1, contained  $10^8$ - $10^9$  bacteria per ml. The plates were incubated as usual, colonies were counted, and their size and intensity of colour estimated.

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TABLE 8

\* on HAP Medium HAP  
\* Carried out at Statens  
and six Selected Atyp

Gonorrhoea strain	Dilut	HAP medium		HAP medium without hemin		Fermentation medium CPH	
		Colony diameter		Colony diameter		Colony diameter	
		smallest mm	largest mm	smallest mm	largest mm	smallest mm	largest mm
A	1/1	1.0	2.0	0.5	1.0	-	-
	1/10	1.0	2.0	0.5	1.0	-	0.2
B	1/1	0.5	2.0	0.5	1.5	-	1.0
	1/10	1.5	2.5	0.5	1.0	-	0.5
C	1/1	0.25	1.0	0.25	0.5	-	-
	1/10	0.25	1.0	-	0.5	-	-
D	1/1	0.5	1.0	-	1.0	-	-
	1/10	0.5	1.0	-	-	-	-
E	1/1	0.5	1.5	-	0.5	-	-
	1/10	0.5	2.0	-	-	-	-
F	1/1	0.5	2.5	0.5	2.0	-	-
	1/10	0.5	2.0	0.5	1.0	-	-
G	1/1	0.2	0.2	0.5	0.5	-	-
	1/10	0.25	0.25	0.5	0.5	-	-
H	1/1	0.2	0.2	0.5	0.5	-	-
	1/10	0.25	0.25	0.25	0.25	-	-
Average value	1/1	0.5	1.3	0.4	0.9	-	(0.1)
	1/10	0.6	1.4	0.5	0.7	-	(0.1)



TABLE 9

Results Obtained from Counting the Number of Colonies on Variants of HAP Medium as Carried out at Statens Serum Institut, Copenhagen, on two Normal Strains (A-B) and six Selected Atypical Strains (C-H) of *V. gonorrhoeae*

Hemin percent Alginate " "		HAP medium		Variants of HAP medium					
		0.003 0.6		0.003				0.6	
<i>V. gonorrhoeae</i> strain	Dilut	Dextrose	Maltose	Dextrose	Maltose	Dextrose	Maltose	Dextrose	Maltose
A	1/1	283	414	451	590	82	138	171	385
	1/10	38	48	49	52	11	18	26	31
B	1/1	680	720	924	700	934	447	107	27
	1/10	76	79	67	84	60	60	5	6
C	1/1	125	145	149	227	78	116	5	16
	1/10	0	21	19	31	1	12	0	1
D	1/1	458	590	514	600	16	45	0	1
	1/10	42	77	64	79	1	2	0	0
E	1/1	526	717	665	1031	14	53	1	0
	1/10	59	72	70	86	2	7	0	0
F	1/1	354	459	600	673	121	457	20	118
	1/10	38	52	96	92	29	11	4	3
G	1/1	1486	1526	1424	1368	1190	1458	1474	1730
	1/10	147	182	161	181	126	212	89	190
H	1/1	452	540	612	510	430	656	374	590
	1/10	32	52	49	48	19	37	24	43
Average number	1/1	546	639	667	720	291	421	269	358
	1/10	55	73	72	82	39	49	19	34

the hemin reduces the number to about half its former value "Fermentation medium CPH", on the other hand, merely produced single colonies of the two normal strains

The measurements of the colonial diameters are reported in Table 8, showing a fairly wide range of 0.25-2.5 mm between different strains, the average being 0.5 mm for the smallest and 1.4 mm for the largest colonies. Here, too, the importance of hemin is illustrated by the fact that only one of the strains developed colonies of a maximum diameter of 2.0 mm in the absence of hemin, the average being only 0.4 mm for the smallest and 0.9 mm for the largest colonies.

The importance of hemin and alginate in different varieties of HAP-medium was also studied—colonies were counted, yielding the results reported in Table 9. Hemin as had already been established was shown to be an essential growth-stimulating factor. In contrast alginate proved to have slightly inhibitory effects, especially pronounced in the medium variety lacking in hemin as well where three strains showed none or very scanty growth. However, the difference between the original HAP-

TABLE 10

Results Obtained from a Determination of the Intensity of Indicator Colour Change on Variants of HAP Medium and Fermentation Medium CPH', as Carried out at Statens Seruminstitut Copenhagen, on two Normal Strains (A-B) and six Selected Atypical Strains (C-H) of *N. Gonorrhoeae*

Hemim Aluminate	per cent	Variants of HAP medium				Ferment medium (PH)
		0.007 0.6	0.073		0.6	
N. gonorrhoeae strain						
A		4	4	2	4	0
B		4	4	4	4	0
C		3	4	0	0	0
D		4	4	0	0	0
E		4	4	0	0	0
F		4	4	2	4	0
G		3	3	3	3	0
H		0	0	0	0.5	0
Average value		3.25	3.4	1.4	1.9	0

medium and the HAP-medium without alginate is small and of no practical consequence

As may be seen from Table 10 the intensity of colour change was given 3 or 4 points on HAP-medium in the case of 7 of the 8 strains. But one of the strains gave no definite colour change. Unfortunately, however, no information is available whether the strain can ferment dextrose and produce a colour change if inoculation is increased so as to give confluent growth.

Dr A. Rejn's investigations resulted in a substitution of HAP-medium, for the previously used "fermentation medium CPH" at the gonococcal department of the Statens Seruminstitut.

In addition to this, a mark, Norway, Finland, a cultivation and resistance tests have recommended a switch on to HAP-medium as a fermentation medium for gonococci.

#### SUMMARY

When new media for the cultivation of gonococci are being worked out, special regard must be paid to atypical strains characterized by scanty growth, small colonies, and poor viability. The

must contain all of the growth-stimu-

TABLE 9

Results Obtained from Counting the Number of Colonies on Variants of HAP Medium, as Carried out at Statens Serum Institut, Copenhagen, on two Normal Strains (A-B) and six Selected Atypical Strains (C-II) of *N. gonorrhoeae*

Hemin percent Alginate " "		HAP medium		Variants of HAP medium					
		0.003 0.6		0.003				0.6	
		Dextrose	Maltose	Dextrose	Maltose	Dextrose	Maltose	Dextrose	Maltose
N. gonorrhoeae strain	Dilut								
A	1/1	283	414	451	590	82	138	171	385
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lating factors needed by normal, and particularly by atypical, gonococcal strains

This new HAP-medium has been tested with 253 consecutively isolated strains of *N. gonorrhoeae* as well as with several, specially chosen, atypical strains of *N. gonorrhoeae*. At the same time, HAP-medium has been compared to "chocolate ascitic agar", "chocolate ascitic fermentation medium", and "fermentation medium CPH". The growth stimulating power of HAP-medium was found to be virtually equal to that of "chocolate ascitic agar" and far superior to that of "fermentation medium CPH". Fermentation reactions are much more readily demonstrable on HAP-medium than on the other two fermentation media.

Thanks to the good growth-stimulating qualities of HAP-medium, the routine procedure was simplified, permitting a finer control of the isolated gonococcal strains. This became possible when it was found that both fermentation and resistance tests could be carried out on the same bacterial suspension, consisting of 10 gonococcal colonies in 1 ml of placenta infusion broth. A loopful of the suspension was streaked across 1/4-1/6 of the four HAP-medium plates with different sugars and the rest of the suspension was poured onto a plate of "chocolate ascitic agar" for resistance test by a diffusion method using 5 different antibiotic discs of paper (2).

This routine procedure thus guarantees that the bacterial strain whose resistance is reported to the clinician, really is *N. gonorrhoeae*, and no fears need arise that strains of lowered susceptibility against penicillin might not be gonococci (4, 19).

Even though long application of HAP-medium may result in the appearance of single strains exhibiting atypical behaviour here too it will be seen from the various test series reported above that this new medium nevertheless has proved its reliability, and its advantages for routine diagnosis if compared to media previously used.

#### ADDENDUM

Investigations were continued on more than 1000 strains of *N. gonorrhoeae* with a view to improve the growth and colour intensity of the very few strains that showed a sparse growth and a weak colour change.

During the routine diagnostic work a few such strains were found and some others were received from other laboratories. In all 12 strains of *N. gonorrhoeae* have been studied which showed a weak or no colour change after an incubation of 20 hours.

All of these strains except three showed a perfect colour change after 48 hours of incubation however.

By the addition of 3 drops of a 0.1 per cent buffered solution of cocarboxylase to the surface of the HAP medium in the area of inoculation the growth and colour

— improved with all of the strains except one

a desirable degree of indicator  
per cent of cocarboxylase was used

Cocarboxylase (Bérolase NORMAN) consisting of 1.0 g sodium acetate, 0.2 g sodium hydroxide and 100 ml sterile water to make a 0.1 per cent solution. Of this solution 6 ml are added to the HAP medium at

These bacteriological properties are phage type drug resistance and production of lipase (Gillespie & Alder 1952). Interest in the last reaction was aroused by the results of *Jessen & al* (1959b), who on investigating staphylococcal bacteraemia cases found that those caused by strains not producing the enzyme (*i.e.* strains that were F<sub>1</sub> negative) had a higher mortality rate than cases caused by strains producing lipase (F<sub>1</sub> positive).

## METHODS

### Registration

The cards have been filed according to the hospital and date of the person's birth. One strain is represented by one card.

The card indicates where the strain was isolated from (nose pathological process etc.) its phage type its drug resistance and its production of lipase.

During the whole period the laboratory received a total of about 10 000 strains isolated from patients or staff members. However in the present survey only one strain from each person is included. The strain chosen is the one first isolated from a pathological process. Strains from nose and throat are included if they represent

### Bacteriological Investigations

Statens Seruminstitut received the specimens either as swabs directly from the hospitals or as pure cultures from other laboratories.

Bacteriophage typing of the staphylococci was performed as described by *Rosenfalck* (1959). Following suggestions from the international committee on phage typing of staphylococci minor changes were introduced. Phages 81 and 834 were included in the basic set of phages. The pooled bacteriophage suspensions employed contain the following phages: Pool A: 31B 42B 41C 57 pool B: 13 724 83B 82B pool C: 44 51 67 78.

Production of lipase was demonstrated as described by *Jessen et al* (1959a) using *Sierra's* medium (1957).

Resistance to antibiotics was determined in several laboratories (Statens Seruminstitut, the University of Copenhagen, Copenhagen, and various hospital laboratories) by the disc method (*Jensen & Kier* 1947).

Resistance to the various antibiotics was reported by *Lund et al* (1951). Strains resistant to the various antibiotics were reported. Strains with a zone diameter were classified as sensitive. If the zone was less than 25 mm the strains were considered resistant.

## RESULTS

The material comprises 6 932 strains isolated from 6 932 persons.

Table 1 shows the distribution of the material over various counties of Denmark.

The figures do not give any information about the frequency of infections or about the occurrence of epidemics. The number of phage typed strains may only reflect local interest in problems concerning hospital infections. Furthermore many strains isolated locally may never have been forwarded to the phage typing laboratory.

The number of specimens received increased in May, 1960. At this time Sykehusforeningen i Danmark set up an organisation to check up

## STAPHYLOCOCCUS AUREUS STRAINS ISOLATED IN DANISH HOSPITALS FROM APRIL 1ST TO DECEMBER 31ST 1960

By

KIRSTEN ROSENDAAL, AKSEL STENDERUP, PETER HELMS  
and KNUD RIHWIERTS ERIKSEN

Received 3 III 61

On April 1st, 1960, Statens Seruminstitut began a registration of all phage-typed *Staphylococcus aureus* (= coagulase-positive staphylococci) strains isolated from patients and staff members of hospitals all over Denmark.

The purpose of this registration was to record the epidemiological occurrence of staphylococci at each hospital. From time to time it proved possible to discover epidemics at their very beginning and to inform the local authorities about the situation.

This arrangement succeeded because the phage-typing of staphylococci was performed in one laboratory only.

The centralization was in certain ways a disadvantage. The registration does not give a true picture of the staphylococcal hospital infections, it was not possible to decide whether the infections from which the staphylococci were isolated originated before or after hospitalization, as the specimens were forwarded to the laboratory without information necessary for making this distinction. Furthermore, it was impossible to ascertain whether all infections of epidemiological interest were bacteriologically investigated.

As a consequence, thorough studies of epidemics and of the frequency of infections could not be carried out on the basis of the present material, which comprises specimens received from April 1st to December 31st, 1960. However, a continued annual survey will permit the observation of changes in the situation from year to year. The registration will continue for at least five years.

In another respect the centralization was a great advantage. It was considered justifiable to regard the strains of the material as epidemiologically unrelated, as they were isolated from about 150 hospitals scattered all over Denmark. Therefore the material is suitable for the evaluation of certain bacteriological properties of the strains investigated—and this is the chief object of the present report.

These bacteriological properties are phage-type, drug resistance and production of lipase (Gillespie & Alder 1952). Interest in the last reaction was aroused by the results of Jessen & *et al.* (1959b), who on investigating staphylococcal bacteraemia cases found that those caused by strains not producing the enzyme (i.e. strains that were EY negative) had a higher mortality rate than cases caused by strains producing lipase (EY-positive).

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was demonstrated as described by Jessen *et al.* (1959a) using *Serratia melioides* (1957).

Resistance to antibiotics was determined in several laboratories (Statens Serum Institut, the Universities of Copenhagen and Århus and various hospital laboratories). Two methods were used: 1) the paper disc method (Jensen & Kier 1947; Dragsted & Frichsen 1953); 2) the tablet method (Lunt *et al.* 1951).

The strains were classified as sensitive or resistant to the various antibiotics investigated. If the size of the inhibition zone was reported, strains with a zone of 25 mm were classified as sensitive. If the zone was less than 25 mm the strains were considered resistant.

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Received 3 vii 62

On April 1st, 1960, Statens Seruminstitut began a registration of all phage-typed *Staphylococcus aureus* (= coagulase-positive staphylococci) strains isolated from patients and staff members of hospitals all over Denmark.

The purpose of this registration was to record the epidemiological occurrence of staphylococci at each hospital. From time to time it proved possible to discover epidemics at their very beginning and to inform the local authorities about the situation.

This arrangement succeeded because the phage-typing of staphylococci was performed in one laboratory only.

The centralization was in certain ways a disadvantage. The registration does not give a true picture of the staphylococcal hospital infections, it was not possible to decide whether the infections from which the staphylococci were isolated originated before or after hospitalization, as the specimens were forwarded to the laboratory without information necessary for making this distinction. Furthermore, it was impossible to ascertain whether all infections of epidemiological interest were bacteriologically investigated.

As a consequence, thorough studies of epidemics and of the frequency of infections could not be carried out on the basis of the present material, which comprises specimens received from April 1st to December 31st, 1960. However, a continued annual survey will permit the observation of changes in the situation from year to year. The registration will continue for at least five years.

In another respect the centralization was a great advantage. It was considered justifiable to regard the strains of the material as epidemiologically unrelated, as they were isolated from about 150 hospitals scattered all over Denmark. Therefore the material is suitable for the evaluation of certain bacteriological properties of the strains investigated—and this is the chief object of the present report.

TABLE 2  
Clinical Sources of Strains Investigated

Group	Isolate from	Number of strains	Percent
A	× unknown source	56	0.8
	• pure culture	33	0.5
	+ swab	227	3.3
	+ pus	934	13.8
	total	1270	18.8
B	++ region stated	439	6.3
	abscesses	1300	18.8
	pustuli	430	6.3
	hands	472	6.8
	cicatrices	344	5.0
	wounds	385	5.5
	combustion	17	0.2
	mastitis	■	1.2
	eczema and skin diseases	12	0.2
	total	3484	50.2
C	eye	119	1.7
	ear	240	3.4
	nose	290	4.2
	mouth and throat	190	2.7
	vagina	24	0.3
	total	863	12.0
D	sinus	23	0.3
	larynx trachea	129	1.9
	bronchi	43	0.6
	sputum	516	7.4
	urine	238	3.4
	feces	31	0.4
	total	1000	14.0
E	blood	63	1.0
F	spinal fluid	9	0.1
	viscera	42	0.6
	exudate	51	0.7
	osteomyelitis	16	0.2
	joint	34	0.5
	+++ fistulae	99	1.4
	autopsy	1	0.0
	total	252	4.0
Total number of strains		6932	100

× (unknown source) No information received  
 • (pure culture) Pure culture  
 + (swab and pus) The only fr  
 ++ (region stated) It is stated  
 been taken  
 excepted  
 +++ (fistulae) Comprises fistulae from viscera and ossa

on hospital infections in Denmark. The increase of specimens may be a result of this new activity. A further increase occurred in September 1960, when it was decided to phage type all *Staphylococcus aureus* strains from hospitals, isolated in the various departments in Statens Seruminstitut. Previously the typing had only been done when requested by the hospitals.

TABLE 1  
*Distribution of the Material on the Various Counties of Denmark*

County	Number of specimens	Population registered July 1st 1960
København (borough)	2403	725 000
Fredrikshavn ( )	214	114 500
København (county)	229	481 000
Roskilde ( )	47	89 800
Frederiksborg ( )	131	180 300
Helsør ( )	79	127 200
Sorø ( )	91	129 800
Præstø ( )	94	122 200
Bornholm ( )	23	45 500
Lolland Falster ( )	86	132 300
Svendborg ( )	26	149 200
Odense ( )	248	264 400
Vejle ( )	221	213 800
Skanderborg ( )	240	137 900
Århus ( )	1446	220 200
Randers ( )	316	170 100
Ålborg ( )	280	238 800
Hjørring ( )	233	177 600
Thisted ( )	83	85 100
Viborg ( )	130	162 000
Ringskøbing ( )	92	205 500
Bjelle ( )	119	185 000
Halslev ( )	70	72 400
Åbenrå Sønderborg ( )	16	105 500
Tønder ( )	4	42 500
Læsø ( )	1	34 500
Grønland ( )	4	31 500
Total	6 932	4581 000

Future registration may reveal whether there is any seasonal fluctuation in the number of staphylococcal cases. An enumeration of the staphylococci isolated at Statens Seruminstitut from May to December 1960 seems to bear out the suggestion that an increase did take place during the autumn of 1960.

The processes and regions from which the staphylococci were isolated are given in Table 2. 50 per cent of the strains originated from various pathological conditions of the skin and the subcutaneous tissues (group B), most of these being from abscesses. At least 5 per cent of the strains were isolated from post-operative infections (e.g. stiches) and

TABLE 4  
Percent Age Distribution of Phage Patterns and Groups among Strains from Various Clinical Sources

Source	Group A	Group B	Miscellaneous	Group C	Group D	Wood	Subtotal	Group A	Total
<i>Phage type group</i>									
80 81	20	21	15	8	5	21	0	17	17
80	3	4	2	1	2	8	0	3	1
81	4	3	5	1	2	2	0	2	1
52/52A/80	15	17	21	10	12	11	33	16	15
421/47C	1	1	0	1	1	0	0	1	1
Total	43	46	63	24	23	38	33	33	39
rest of group I	9	7	8	8	4	8	11	5	7
total of group I	52	53	72	32	27	45	44	44	46
total of group II	18	25	12	22	15	17	11	11	16
total of group III	15	14	9	21	20	17	11	22	17
83A	3	3	0	2	9	7	11	7	1
miscel total	5	5	2	6	12	6	11	8	6
NI	2	3	1	1	4	0	0	1	1
NT	11	10	4	15	14	14	22	12	11
Total number of strains	1270	3399	83	863	1000	211	9	242	6932

Only 1 strain from autopsy is included in the survey

No representatives of group IV were found

For an explanation of the subdivision of the clinical sources see Tables 2 and 3

52/52A/80\* comprises strains of phage types 52/80 52/52A/80 52/52A/80 81 52/80/81 etc

TABLE 3  
*Strains from Various Human Sources in Relation to Age-Groups (Percentage Distribution)*

Age	Year of birth										1870-90	
	0	10	20	30	40	50	60	70	80	90	1870-90	1890-1900
<i>(Line of source)</i>												
Group A	12	17	24	23	20	18	14	16	12	19		
Group B	43	43	50	47	53	50	49	50	54	49		
Mastitis	1	0	2	4	2	0	0	0	1	1		
Group C	37	29	15	17	9	6	4	6	10	13		
Group D	3	7	5	7	11	21	28	22	19	14		
Blood	0	0	1	0	1	1	2	2	1	1		
Group E	4	4	3	2	4	4	3	4	3	3		
Total	100	100	100	100	100	100	100	100	100	100	100	100
Total number of strains	323	603	828	974	698	771	865	695	223	6932		

Specimens from spinal fluid and ulcers were below 1 per cent each  
 % = percentage of total number of strains

A specific survey of the clinical sources is seen from Table 2

Group A comprises mostly strains from unspecified sources

Group B comprises mostly strains from skin and subcutaneous tissues

Group C comprises mostly strains from bodily orifices

Group D comprises mostly strains from excreta

Group E comprises mostly strains from tissues and bodily fluids, having no connection with the surface of the body

TABLE 4  
Percentage Distribution of Phage Patterns and Groups among Strains from Various Clinical Sources

Percentage Distribution of Phage Types among Strains from Various Sources															
Source	Group A		Group B		Stillis		Group C		Group D		Group E		Total		
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	
Phage type group															
80/81	20		21		75		8		5		21		0		
80	8		4		2		1		3		3		0		
81	4		3		5		3		2		2		0		
52/52A/80	15		17		23		10		12		11		73		
42/42C	1		1		0		1		1		0		0		
total	43		46		113		24		23		18		33		
rest of group I	0		7		8		8		4		8		11		
total of group I	52		53		72		32		27		45		44		
total of group II	18		25		12		22		15		17		11		
total of group III	15		14		9		21		29		17		11		
83A	1		3		0		2		9		3		11		
miscel total	5		5		2		7		12		6		11		
NI	3		3		1		1		4		0		0		
NT	11		10		4		15		14		14		32		
Total number of strains															
	1270		3399		85		863		1000		63		9		
														242	6032

Only 1 strain from autopsy is included in the survey

No representatives of group IV were found

For an explanation of the subdivision of the clinical sources see Tables 2 and 3  
52/52A/80 comprises strains of phage types 52/80 52A/80 52/52A/80 52/80/81 52/80/81 etc

TABLE 5  
Antibiotic Resistance of the Various Phage Types/Groups

Antibiotic phage types/groups	Penicillin			Streptomycin			Tetracyclines			Chloramphenicol			Frythromycin			Total				
	num ber	res	res %	num ber	res	res %	num ber	res	res %	num ber	res	res %	num ber	res	res %					
80/81	429	354	83		426	371	87		410	20	5		409	8	2		216	2	1	1160
80	135	107	79		135	121	90		132	4	3		132	1	1		55	0	0	226
81	81	60	74		81	53	66		80	12	15		80	2	3		42	0	0	225
52/52A/80	521	382	73		516	365	71		512	53	10		511	16	3		221	4	2	1045
42B/47C	40	31	78		39	10	26		39	4	10		39	1	3		25	0	0	81
rest of group I	218	62	28		217	22	10		217	5	2		217	3	1		103	2	2	480
3A	200	104	52		197	6	3		197	6	3		196	6	3		84	0	0	393
71	16	9	56		25	1	4		25	1	4		25	0	0		13	0	0	53
rest of group II	328	103	31		323	6	2		325	9	3		325	6	2		177	1	1	681
group III	555	241	43		548	128	23		548	79	14		546	29	5		255	7	3	1168
group IV	2	0	0		2	0	0		2	1	0		2	0	0		2	0	0	5
83A	117	104	89		117	110	94		117	105	90		113	4	4		52	3	6	242
rest of mixed	87	17	19		84	6	7		84	2	2		85	2	2		45	0	0	160
mixed group	104	54	52		104	23	23		104	20	20		104	4	4		47	0	0	208
NT	418	187	61		412	103	25		412	63	15		412	23	6		185	0	0	776
Total	7251	1898	58		3226	1325	41		3204	384	12		3196	105	3		1528	19	1	8932

another 5.5 per cent may be added to this group if strains from wounds (this designation may comprise cicatrices, accidental wounds and ulceration of the skin) are added. In 18.2 per cent of the specimens the origin was unknown (referred to in the table as unknown, pus, swab, pure culture = group A).

Table 3 shows the number of staphylococcal strains isolated from various human sources in relation to age groups. The first column indicates the number of strains from children up to 1 year old. All the other columns indicate the number of isolates from patients in the age groups 1-10 years, 10-20 years etc.

323 specimens from children up to 1 year old seems a remarkably high figure. When the other figures in the table are compared, it will be noted that the highest number of strains originated from patients between 20-30 years old. About the same number were isolated from patients 50-60 years old.

As might be expected, strains from the skin are predominant in all age groups. For persons under 30 years, specimens from "bodily orifices" (group C) come next, for groups over this age, strains from excretes (group D).

### Phage Types

Tables 4 and 5 show that 'type 80', comprising types 80, 81, 80/81, 82, 84/80 and 42B/47C, is the one most frequently found. It is predominant in all diagnostic groups, especially among mastitis strains. The excretes are an exception, here strains belonging to group III form the majority. In the total material, group III and group II strains represent 18 and 16 per cent respectively.

In the total material the "new type" 83A amounts to only 3 per cent. Table 6 gives specified information about its occurrence. Most frequently it has been isolated from excretes and exudates.

11 per cent of the strains were non typable (NT). On the whole, non-typable strains are evenly distributed over the various sources, the only exception being the mastitis strains, the percentage of which is below the average.

### Antibiotic Resistance Correlated with Phage Types

Only for half of the strains is there any information about resistance to antibiotics (Table 7).

Of the strains which are penicillin-resistant, about 40 per cent resistant to streptomycin, and only 14 per cent resistant to tetracyclines. Resistance to chloramphenicol and erythromycin is very rare (3 per cent and 1 per cent respectively).

Furthermore, it is obvious (Table 5) that there is a certain correlation between phage types and resistance to antibiotics. In fact the



greatest number of "type 80" strains are resistant to penicillin and streptomycin, very few are resistant to tetracyclines.

Type 83A is almost constantly resistant to penicillin, streptomycin and tetracyclines, and the majority of all the strains resistant to these antibiotics belong to type 83A (Table 7).

About half the strains of phage-groups III, NI and NT are penicillin resistant, about 20 per cent are streptomycin-resistant and comparatively many strains are resistant to tetracyclines.

Many strains sensitive to all antibiotics are found among representatives of phage groups I ("type 80" excepted) and II. However, about 50 per cent of type 3A strains (group II) are penicillin-resistant.

TABLE 6  
Occurrence of Phage Type 83A

Isolated from	Total number of strains	Phage type 83A	
		total number	per cent
unknown source	56	2	3.6
region stated	439	12	2.7
pus	954	29	3.0
swab	227	7	1.3
abscesses, etc	1300	15	1.2
pustuli etc	430	9	2.1
hands	472	2	0.4
scars	344	22	6.4
wounds	785	25	6.5
combustion	17	1	5.9
eczema	12	1	8.4
eye	119	1	0.8
ear	240	6	2.5
nose	290	4	1.4
mouth	190	2	1.1
vagina	24	1	4.2
larynx, trachea	129	10	7.8
bronchii	43	5	11.2
sputum	516	41	8.0
urine	258	28	10.9
faeces	31	4	12.9
blood	63	2	3.2
spinal fluid	9	1	11.0
viscera	42	3	7.1
exudate	51	7	13.8
fistulae	99	6	6.1
autopsy	1	0	0
total of phage typed strains	6332	242	3.5

Type 83A was not found in any specimen from mastitis, sinus, osteomyelitis and joint disease cases.

### Lipase Production

24 per cent of 6,890 strains investigated could not be shown to produce lipase (Fig. 1).

TABLE 7

*Occurrence of Strains Resistant to Penicillin Streptomycin and Tetracyclines within the Various Phage Types Groups*

Phage type group	Number of strains investigated	Number of strains resistant to P+S+T	
		Total	Per cent
80 81	410	19	4.6
80	132	1	0.8
81	80	6	7.4
52 52 V/80	512	34	6.6
42II 47C	39	2	5.1
rest of group I	217	2	0.9
total of group I	1390	64	4.6
3A	197	1	0.5
71	24	1	4.0
rest of group II	325	1	0.3
total of group II	547	3	0.6
total of group III	548	59	10.7
total of group IV	2	0	0
83A	117	97	81.1
rest of miscel	84	2	2.3
total of miscel	201	97	48.2
mixed group	104	16	15.4
NT	412	49	11.9
total	3201	288	9.2

P = penicillin

S = streptomycin

T = tetracyclines

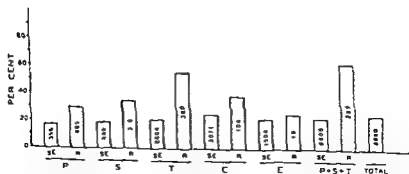


FIG. 1

Occurrence of H negative strains related to antibiotic resistance. The figures in the columns indicate number of strains. P = penicillin S = streptomycin T = tetracyclines C = chloramphenicol E = erythromycin SE = sensitive R = resistant

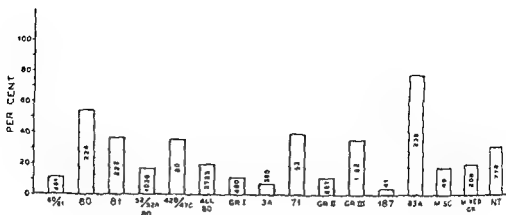


Fig 2

Occurrence of EY negative strains, related to phage types/groups  
The figures in the columns indicate number of strains

The occurrence of EY-negative strains was unevenly distributed throughout the material, they were more numerous among antibiotic-resistant strains than among sensitive ones. 61 per cent of staphylococci resistant to penicillin, streptomycin and tetracyclines were EY-negative. Thus, the percentage of EY-negative strains also is highest among types with the highest developed resistance to antibiotics (Fig 2) 79 per cent of strains belonging to type 83A are EY-negative "Type 80" taken as a whole is an exception. Only 20 per cent of this type are EY-negative. However, in this respect the various representatives within the type behave differently. Types 80 81 and 52/52A/80 have very few EY-negative strains, whereas the other members of the "type" 80, 81 and 42B/47C, are often EY-negative.

## DISCUSSION

In one respect the present material is unusual. It represents a survey of a large number of epidemiologically unrelated phage-typed *Staphylococcus aureus* strains collected from about 150 Danish hospitals during the last nine months of 1960.

We have not been able to find any other material directly comparable to the present one. Those we have considered all deal with strains collected over several years (Blair & Carr 1958) and isolated in a single hospital where it has been possible to make a distinction between hospital cases and community cases (Bynoe, Elder & Combs 1956, Spink 1956, Bauer, Perry & Kirby 1960, Wallmark & Finland 1961) in one respect all these studies disagree with ours in that the resistant strains are less numerous in the Danish material, in particular, strains resistant to tetracyclines, chloramphenicol and erythromycin are seldom encountered by us.

"Type 80" which in other parts of the world, too, is the most widespread "type", is rarely reported as being sensitive to tetracyclines,

whereas the Danish representatives of the type are, as a rule, resistant to penicillin and streptomycin only. We do not know whether a more cautious Danish policy as regards the use of antibiotics may account for the divergent antibiotic resistance of the staphylococci suggested by Bauer, Perry & Kirby (1960) in a report from an American hospital.

Williamis (1960) mentions that type 83A has recently become epidemic in England. In non-European countries it is not possible at the present moment to follow the trail of this type, because phage 83A has not been widely used for typing. We are of the opinion that this "new type" is becoming more frequent in Denmark, for in 1961 it caused two minor hospital epidemics. As this type combines multi-resistance to antibiotics (to penicillin, streptomycin and tetracyclines) with the possible virulence factor, EY-negativity, it may cause infections difficult to treat and with uncertain outcome. Therefore, the wider spread of this type is being closely studied by the present authors.

Rather few EY-negative strains are found among representatives of type 80/81. This may probably be explained by the fact that a large percentage of these strains in the present material were isolated from abscesses and furunculi, where EY-positive staphylococci are especially frequently encountered (Gillespie *et al.* 1952, Jessen *et al.* 1959a).

During recent years, Jessen *et al.* (1959a, 1963) have investigated staphylococci isolated from bacteraemia cases. The bacteriological findings of these reports are very similar to those of the present paper, but more penicillin-resistant strains were found among the strains causing bacteraemia. A comparison between the two materials is made in another report contained in this issue (Jessen *et al.*, p. 85).

## SUMMARY

On April 1st, 1960, Statens Seruminstitut, Copenhagen, began a registration of all phage-typed *Staphylococcus aureus* strains isolated from persons—patients and staff—connected with Danish hospitals.

During the last nine months of 1960, 6,932 persons from about 150 hospitals were registered.

This report comprises a survey of one strain—preferably from pathological processes—from these persons. It was found that about 50 per cent of the strains were isolated from pathological conditions of the skin and at least 5 per cent from post-operative infections.

"Type 80" was the type most frequently found. Most of these strains were resistant to penicillin and streptomycin only. 3 per cent of the strains belonged to a "new" type, 83A, which was almost constantly resistant to penicillin, streptomycin and tetracyclines. 11 per cent were non-typable.

Only for half of the strains was there any information about resistance to antibiotics. 50 per cent were resistant to penicillin, 40 per cent to streptomycin and 14 per cent to tetracyclines.

24 per cent of the strains were lipase-negative (LY-), the percentage being highest among antibiotic-resistant strains

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## SOME PROPERTIES OF STAPHYLOCOCCUS AUREUS, POSSIBLY RELATED TO PATHOGENICITY

*Part 3: Bacteriological Investigations of Staphylococcus aureus Strains  
from 462 Cases of Bacteraemia*

By

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In two preceding papers (Jessen, Faber, Rosendal, Eriksen 1959a, b) an attempt was made to elucidate possible relationships between pathogenicity and some measurable *in vitro* properties of *Staphylococcus aureus*, by correlating the latter with the clinical type and course of infection in a number of human cases (bacteraemia, boils and impetigo lesions).

This approach demands a large material to reduce the influence of many variable, unrecorded or unknown factors. For that reason, and in order to detect possible changes in the characters of the prevalent strains, some of the investigations from the years 1957-58, namely those on bacteraemia cases, were continued during the following two years. The bacteriological observations in a total of 462 epidemiologically unrelated cases of staphylococcal bacteraemia from these four years are reported here.

The following properties were investigated: phage type, virulence, mortality rate, egg count, hospital origin of the infections, and serious primary diseases of the patients. No immediate correlation was established between mortality rate and phage type, production of alpha lysin or production of hyaluronidase.

The two latter properties were omitted from the present investigations.

Previously it had been found (Jessen, Faber, Rosendal & Eriksen 1959a) that the strains causing bacteraemia in some respects represented an inhomogeneous group in contrast to those isolated from impetigo lesions and boils. In order to see if bacteraemia strains differ from strains from other sources, the present report includes a comparison

24 per cent of the strains were lipase-negative (LA-), the percentage being highest among antibiotic-resistant strains

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## SOME PROPERTIES OF STAPHYLOCOCCUS AUREUS, POSSIBLY RELATED TO PATHOGENICITY

Part 3<sup>1</sup> Bacteriological investigations of *Staphylococcus aureus* Strains  
from 462 Cases of Bacteraemia

By

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In two preceding papers (Jessen Faber Rosendal Eriksen 1950a b) an attempt was made to elucidate possible relationships between pathogenicity and some measurable *in vitro* properties of *Staphylococcus aureus* by correlating the latter with the clinical type and course of infection in a number of human cases (bacteraemia boils and impetigo lesions).

This approach demands a large material to reduce the influence of many variable unrecorded or unknown factors. For that reason and in order to detect possible changes in the characters of the prevalent strains some of the investigations from the years 1947-58, namely those on bacteraemia cases, were continued during the following two years. The bacteriological observations in a total of 162 epidemiologically unrelated cases of staphylococcal bacteraemia from these four years are reported here.

The two preceding papers indicated a correlation between high mortality rate and the following factors: Antibiotic resistance and negative coagulability of the strains, hospital origin of the infections and serious primary diseases of the patients. No immediate correlation was established between mortality rate and phage type, production of alpha toxin or production of hyaluronidase.

The two latter properties were omitted from the present investigations.

Previously it had been found (Jessen Faber, Rosendal & Frisken 1951) that the strains causing bacteraemia in some respects represented an inhomogeneous group in contrast to those isolated from impetigo lesions and boils. In order to see if bacteraemia strains differ from strains from other sources the present report includes a compa-



parison between bacteraemia strains and a large material of staphylococci as regards phage-type, resistance to antibiotics and occurrence of  $\Phi$ -negative strains. This large material was isolated from specimens from Danish hospitals during the last 6 months of 1960, and a survey is published elsewhere in this issue (Rosendal, Stenderup, Helms & Eriksen 1963).

Recently Moore (1960) demonstrated a bimodal distribution of *Staphylococcus aureus* strains when they were characterized according to the concentration of mercuric chloride required for complete growth inhibition. From concurrent clinical experience he advanced the hypothesis that a relation exists between mercury-resistance and the epidemic character of the strains. Because of this observation the mercury resistance of all the bacteraemia strains has been investigated.

### MATERIAL

The material consists of 497 strains of coagulase positive *Staphylococcus aureus* strains isolated at Statens Seruminstitut and the Blegdamshospital by blood cultures from 462 patients with bacteraemia. This number comprises 201 previously reported cases from 1957-58 and 261 cases from the years 1959-60. Clinical information in all but 6 cases was obtained from the hospitals concerned<sup>1</sup>. The sex and age of the patients are given in a paper dealing with some clinical aspects of the cases (Rosendal, Faber, Høne, Jessen & Eriksen 1962). The laboratory examination of a few strains is incomplete in one or several respects which explains minor differences in the total number of strains in the tables.

### METHODS

Phage typing, antibiotic sensitivity tests and egg yolk reaction were performed as described previously (Jessen, Faber, Rosendal, Eriksen 1959a). Resistance to mercuric chloride was examined by the method given by Moore (1960) using freshly prepared agar plates containing  $\text{HgCl}_2$  in a concentration of 1/75000 and inoculated spotwise with one loopful of an 18 hrs. broth culture. The results were recorded after 48 hours' incubation at 37° C.

### RESULTS

#### *Frequency of Staphylococcal Bacteraemia*

The number of cases diagnosed in each of the four years 1957-60 was 101, 100, 138, and 123 respectively. These figures are not assumed to reflect a real increase in the frequency of bacteraemia within the four-year period, but a stimulated interest in bacteriological investigations as the number of blood specimens examined per year increased by 50 per cent during the period. For the whole period, however, staphylococcal bacteraemia seems more common than in the preceding four-year period (Jessen, Faber, Rosendal 1959).

Hospital infections accounted for 50 per cent of the cases in 1957, the percentage decreased to 43 in 1958, 41 in 1959 and 37 per cent in 1960.

<sup>1</sup> Our thanks are due to numerous hospital departments for placing clinical data at our disposal.



The increase in the number of tetracycline-resistant strains can be partly ascribed to the occurrence of strains of type 83A. Out of 16 strains of this type 14 were tetracycline-resistant.

Five strains only were resistant to chloramphenicol, and seven to erythromycin.

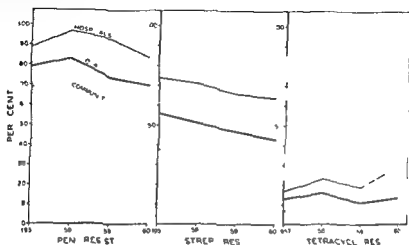


Fig 1

Strains resistant to penicillin streptomycin and tetracyclines in the years 1957-60 (percentage distribution)

TABLE 2  
*Phage-Types and Antibiotic Resistance*

Phage group or type	Number of strains	Penicillin resistant per cent	Strepto mycin resistant per cent	Tetra cycline resistant per cent	Chloram phenicol resistant	Erythro mycin resistant
80/81	132	97	86	6	0	3 strains
52/52A/80/81	57	89	85	4	2 strains	1 strain
Gr I others	36	58	22	6	0	0
Group II	63	40	3	5	0	0
Group III	113	81	22	29	3 strains	1 strain
NI + Miscel	28	64	32	25	0	1 strain
NT	49	59	39	37	0	1 strain

Antibiotic resistance of 478 bacteraemia strains NT = non typable NI = not identified

### Mercury-Resistance

The mercury resistance was investigated in 430 strains. The incidence of resistant strains was very constant through the four years 43, 46, 46, and 45 per cent respectively.

There is a pronounced correlation between antibiotic resistance and mercury-resistance in so far as strains sensitive to all antibiotics practically always were sensitive to mercury as well. Among strains resistant to penicillin but sensitive to other antibiotics one fifth were mer-

TABLE 3  
Resistance to Mercuric Chloride Related to Phage-Type and Antibiotic Resistance

	Resistant to	T per cent	P only per cent	P+S only per cent	P+S+T only per cent	S only per cent	T only per cent
Hg resistant	80/81 and 52/52 A 80/81	0 — (0/8) 3 — (2/80)	81 — (13/16) 10 — (10/102)	85 — (115/135) 41 — (9/22)	86 — (9/7) 75 — (24/32)	(1/1) (0/2)	(0/0) 63 — (5/8)
Other types							
Total		2 — (2/88)	20 — (23/118)	86 — (124/157)	77 — (90/99)	(1/3)	63 — (5/8)

P — penicillin  
S — streptomycin  
T — tetracycline

The numerator indicates number of Hg resistant strains, the denominator indicates the total number of strains.

cury resistant, and among those resistant to penicillin and streptomycin four fifths were resistant (Table 3)

However, this concordance between resistance to antibiotics and Hg resistance to a certain extent reflects another correlation, viz between Hg resistance and phage-type. If strains having identical resistance are considered it is evident from Tables 3 and 4 that certain phage-types are mercury-resistant far more often than others with the same antibiotic resistance. It is most easily seen for the types 80/81 and 52/52A/80/81 (Table 3) and for type 83A (Table 4)

TABLE 4  
*Proportion of Hg Resistant Strains in Different Phage Groups*

Phage type or group	P sensitive	P resistant			
		Total		Resistant to P and S	
	Number	Number	Per cent	Number	Per cent
80/81	1/3	97/114	85	83/99	84
52/52A/80/81	0/6	39/46	85	38/43	88
Gr I others	0/14	7/21	33	6/8	75
Group II	1/32	1/24	4	0/0	
Type 83A	0/0	13/15	87	13/13	100
Gr III others	4/21	16/77	21	4/16	25
NI + Miscel	1/8	5/14	36	1/4	25
NT	1/15	9/20	59	9/11	82
Total	8/99 = 8%	187/331	58	154/194	79

A total of 430 strains investigated for Hg resistance. In this table type 83A strains have been removed from the groups III, NI and NT and listed separately.

P = penicillin S = streptomycin NT = non typable NI = not identified

Mercury-resistance was more frequent in hospital strains (58 per cent) than in community strains (36 per cent). This distribution was to be expected because of the predominance of antibiotic resistant strains and of strains of phage type '80' and 83A in hospitals. The difference is greatly reduced if only penicillin resistant strains are considered (Hospital 59 per cent, community 54 per cent) and it disappears entirely if strains of the same phage type are compared. Thus, the difference in Hg reaction between hospital and community strains may be entirely secondary to these two other factors.

Correspondingly, no direct correlation could be established between Hg resistance on one side, and on the other side the portal of entry of the infections, the egg yolk reaction of the strains or in 150 strains investigated—the amount of hyaluronidase or of alpha lysin produced.

### *Egg-Yolk Reaction*

The frequency and distribution of the EY-negative strains corresponded to that found previously (Jessen, Faber, Rosendal & Eriksen

1959a) The negative EX-reaction was most common among penicillin- and streptomycin resistant strains of phage-group I and III, and among hospital strains

### *Bacteraemia Strains Compared with 6932 Strains from Miscellaneous Sources*

The latter strains have been isolated from a variety of clinical sources including skin, noses etc (Rosendal, Stenderup, Helms & Eriksen 1963) during 1960. As they have been collected from about 150 hospitals all over Denmark, they have been considered epidemiologically unrelated

TABLE 5  
*Phage Types of Bacteraemia Strains as Compared with a Material of Strains from Other Sources*

		80-81 per cent	52-53 & 80-81 per cent	group 2 others per cent	group III per cent	group III per cent	NT per cent	SA per cent	SA+miscellaneous per cent
Bacteraemia	488 strains	28	12	7	13	24	11	3	3
Miscellaneous	6932 strains	23	15	7	16	17	11	3	6

From Table 5 it is seen that the phage-types of the two materials correspond closely to each other. The same is true of resistance to streptomycin and tetracyclines, whereas the miscellaneous material comprises a relatively greater number of penicillin-sensitive strains than does the bacteraemia material, also when strains exclusively from 1960 are compared (Table 6).

LA-negative strains are encountered with the same frequency in both materials, about 25 per cent in each.

TABLE 6  
*Antibiotic Resistance of Bacteraemia Strains as Compared with a Material of Strains from Other Sources*

Resistant to	Penicillin	Streptomycin	Tetracyclines
	Per cent	Per cent	Per cent
Bacteraemia total (488 strains)	76	49	12
Bacteraemia 1960 (120 strains)	70	43	13
Miscellaneous 1960 (3251 strains)	58	11	12

### *Mortality Rate Dependence on Host Factors and Origin of the Infection*

Mortality rate was slightly lower in 1958-60 than in 1957 (Table 7) in all groups of patients, i.e. irrespective of the origin of the infection,

TABLE 7  
*Mortality through 4 Years*

	1957	1958	1959	1960	Total
	Per cent	Per cent	Per cent	Per cent	Per cent
Hospital	59	51	62	51	53
Community	35	22	27	26	27
Total, incl. unknown origin	48	35	39	35	39

the antibiotic resistance of the strains, and other factors considered

About one fifth of the patients infected outside hospitals, and two fifths of the hospital-infected patients were considered especially prone to develop serious infections because of primary diseases such as neoplasms, severe blood disorders, cirrhosis of the liver, diabetes, and severe renal failure. The overall mortality rate in this group of patients was 54 per cent as against 33 per cent for patients without these complications.

The group of hospital-infected patients comprises many of these bad risks; however, this fact does not fully explain the great difference in mortality rate between the hospital group and the community group (Table 8) as the difference is especially pronounced in patients without serious complications.

TABLE 8  
*Serious Pre Existing Conditions Influencing Mortality*

Origin	Complicating conditions present		No pre existing complications		Total mortality per cent
	Number	Mortality per cent	Number	Mortality per cent	
Hospital	81	60	114	49	53
Community	40	53	174	21	27
Total incl. unknown origin	137	54	320	31	39

### *Mortality Rate Relation to Antibiotic Resistance*

In the following sections cases due to more than one strain are omitted. The figures referred to are given in Table 9.

The mortality rate for different groups of patients varied from 21 per cent for those infected outside hospitals with penicillin sensitive strains to 67 per cent for patients infected with streptomycin-resistant—and that is, practically always, also penicillin resistant—hospital strains.

For patients infected with penicillin- and streptomycin-sensitive strains the origin of the infection was of minor importance for the prognosis, but for infections due to resistant strains the above-mentioned difference between hospital and non-hospital strains is striking.

The total material shows a mortality rate for infections due to penicillin- or streptomycin-resistant strains more than twice as high as that for infections due to sensitive strains

TABLE 9  
*Antibiotic Resistance of the Infecting Strains Correlated to Mortality*

Origin	Penicillin		Streptomycin		Tetracyclines	
	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
Hospital	59 (162)	24 ( 17)	67 (123)	30 ( 56)	65 (37)	54 (132)
Community	31 (127)	21 ( 76)	34 ( 64)	24 (139)	33 ( 9)	27 (194)
Total	46 (321)	22 (104)	55 (209)	24 (216)	54 (50)	39 (375)
incl unknown						

Mortality in 425 cases divided into groups according to the origin of the infection and the antibiotic resistance of the strain. Mortality is given in per cent. The number of cases in each group is indicated in brackets. In the total group is included 42 cases of unstated origin.

### *Mortality Rate Relation to E1 Reaction*

Infections due to EY- and E1+ strains had a mortality rate of 58 and 38 per cent respectively. However, the proportion of EY- strains is not uniform in all groups of the material. It is highest in hospital strains resistant to penicillin and streptomycin. Therefore a possible relation between mortality and EY reaction must be evaluated in groups where the strains are of uniform origin and antibiotic resistance. Other factors influencing mortality, such as the presence of complications, the age of the patients, and the portal of entry of the infections, could be shown not to influence the conclusions, neither could they be expected to do so, as the proportion of EY- strains was fairly uniform in groups established along these lines of division.

TABLE 10  
*Mortality and E1 Reaction in Comparable Groups of Patients*

	Hospital		Community		Hospital		Community	
	E sens	E res	E sens	E res	E sens	E res	E sens	E res
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
E1	29 (14)	56 (92)	30 (46)	26 (97)	29 (38)	65 (71)	20 (104)	33 (49)
E1	41	67 (63)	30 (13)	52 (21)	46 (13)	72 (56)	39 ( 23)	45 (11)

Percentages indicate mortality rate.

Total number of cases in each group is given in brackets.

The required comparison is carried out in Table 10. The splitting up of the material into eight groups makes the figures small, and very considerable differences in mortality are required to obtain statistically significant results. However, the results are sufficient to call for further



investigations of possible fundamental differences between EY- and EY+ strains. In each of the eight groups established in Table 10 mortality rate is highest for infections due to EY- strains. Furthermore, for the groups of community infections the mortality rates increase through the following groups: EY+ sensitive, EY+ resistant, EY- sensitive, EY- resistant. For the hospital infections the group of sensitive EY- strains is small or non-existent.

In the total material 40 per cent of 211 patients infected with EY+ penicillin resistant strains died, whereas the mortality rate among 93 patients infected with EY- penicillin resistant strains was 62 per cent.

### *Mortality Rate Relation to Phage-Type*

An overall estimation will charge infections due to certain phage types, e.g. 80/81, with a higher mortality rate, and infections due to other types, e.g. group II strains, with a mortality rate lower than the average (Table 11, last column). This does not necessarily imply that e.g. a type 80/81 strain *per se* is more virulent in the clinical situation investigated than any other strain. The difference seems to reflect two more important factors: The prevalence of type 80/81 strains in the hospital infection group, and of group II strains in the community group, and the generally higher antibiotic resistance of the former type of strains, which might have caused therapeutic failure in a great number of cases.

TABLE 11  
*Mortality and Phage Type of the Infecting Strains*

Origin of infection	Hospital		Community		Hospital + (community + unknown)
	Resistant		Sensitive		Resistant + sensitive
	Per cent	Per cent	Per cent	Per cent	Per cent
Penicillin sensitivity					
80/81	66 (62)	11 (2)	32 (46)	0 (2)	39 (131)
52/52A/80/81	57 (30)	0 (2)	31 (16)	25 (4)	44 (55)
Gr. I others	70 (10)	100 (1)	43 (7)	11 (9)	44 (32)
Group II	0 (6)	40 (5)	17 (12)	20 (24)	19 (53)
Group III	55 (29)	20 (5)	33 (13)	15 (13)	39 (92)
NI + miscel.	67 (9)	(0)	0 (6)	17 (6)	36 (22)
NT	56 (16)	0 (1)	50 (4)	16 (11)	41 (41)

Mortality rate is given in percentages. Total number in each group is given in brackets.

It is convincingly shown in Table 11 that when the origin of the infection, and the antibiotic resistance of the strains are taken into consideration, no difference in mortality rate can be said to depend upon difference in the phage-type of the infecting organism.

### Mortality Rate Relation to Mercury Resistance

The present material confirms the observation (Moore 1960) that mercury resistance is especially characteristic for antibiotic resistant strains common in hospitals and belonging to certain phage types known for their epidemic occurrence. Some of the possible explanations have been discussed by Moore. One possibility is a difference in pathogenicity between mercury resistant and sensitive strains causing a selection of the mercury resistant strains in hospitals. Although the present material permits an evaluation of a small facet of pathogenicity only the property was included in the investigations.

Regarding the material as a whole mortality rate for infections due to Hg resistant strains is 50 per cent as against 33 per cent for infections with Hg sensitive strains. This difference reflects the preponderance of antibiotic resistant strains in the group of Hg resistant strains as shown in Table 3. From the table it appears that practically no antibiotic sensitive strains were Hg resistant.

TABLE 12  
*Mortality Rate to Mercury Resistance of the Strains*

	Origin	Hg resistant	Hg sensitive
		Per cent	Per cent
1 Total material	not considered	50 (182)	33 (201)
Penicillin sensitive	not considered	0 (0)	33 (86)
Penicillin resistant	not considered	51 (177)	41 (115)
Penicillin resistant	Hospital	65 (96)	52 (34)
Penicillin resistant	Community	31 (64)	29 (52)
Streptomycin sensitive	Hospital	30 (30)	27 (165)
Streptomycin resistant	Hospital	54 (152)	56 (36)

The figures in brackets indicate total number of strains investigated

When the material is split up according to penicillin resistance (Table 12, 2) the difference in mortality rate is greatly reduced and found only in the group of infections due to penicillin resistant strains. In part 3 of the table the difference is referred to the penicillin resistant hospital strains but when the groups of hospital strains are divided according to streptomycin resistance mortality rate is unrelated to the Hg resistance of the strains (part 4).

### DISCUSSION

Any relation between biochemical properties of micro organisms and their pathogenicity for man must be confirmed by a series of experiments. This is which is

In the present study it is attempted to avoid the bias of clinical evaluation of the severity of the infections by defining strictly the facts observed and the conditions studied. The measure of pathogenicity is the death or survival of the patient in the situation where staphylococci have been demonstrated to gain entrance to the bloodstream.

As a consequence the material comprises cases of acute fulminating septicæmia almost sure to run a fatal course, as well as cases of bacteraemia with less serious symptoms. This is no inconvenience, but a crucial point in the study, the very aim of which is to elucidate whether such differences are determined largely by host factors such as age, primary diseases, portal of entry etc., or whether they can be correlated to measurable bacterial properties.

A common source of error has been avoided by collecting the material from the whole country so that no immediate epidemiological relation exists between the strains.

Nevertheless the method is open to criticism. The numerous factors which are scrupulously considered and standardized in animal experiments are to a great extent unknown or variable here. By means of the clinical information and by dividing the material along many different lines we have tried to point out some of the important factors influencing prognosis and explain their interdependence. These factors include the origin of the infection, i.e. whether the infection was contracted in or outside hospital, the presence of serious primary diseases, and the antibiotic resistance of the strains.

The last-mentioned factor can be assumed to influence mortality primarily by reducing the chances of effective antibiotic treatment. The question whether the antibiotic-resistant strains beyond this *per se* are more virulent than the sensitive ones can be answered only by examining a material of untreated cases. Comparison between about 3000 miscellaneous strains and the bacteraemia strains does not indicate that the strains causing bacteraemia represent a selection of the resistant strains.

The confirmation of the connection between negative *ES* reaction and high mortality suggests that further work on the nature of this reaction and its connection with other biochemical or physical properties might provide valuable information. Because of the considerable number and the serious character of the infections due to *LA*-negative strains the proposal to limit phage-typing to *LA* positive strains in epidemiological investigations (Graber *et al.* 1958) must be rejected in any case the use of this character in the selection of strains on primary isolation (Carter 1960) is unfortunate, especially as we have recorded some hospital epidemics due to *LA*-negative strains. Investigations of strains in serial subcultures and of 25 colonies of each of 109 bacteraemia strains, and of strains isolated successively from the same patient have indicated that the reaction is sufficiently stable to be of use in epidemiological investigations. It is of special use in the analysis of

epidemics due to very common types where the phage type alone is not sufficient indication of epidemiological connection

Supplementary epidemiological information may be provided by the *Hg* reaction as it is independent of the *EY* reaction and is regarded as reasonably constant. Our investigations confirm *Moore's* (1960) observation of a bimodal distribution of the strains and confirm that the majority of the antibiotic resistant strains of phage types 80 81, 52 a2A/80 81 83A and certain other types are *Hg* resistant. The present material cannot provide evidence in favour of or against *Moore's* suggestion of a connection between *Hg* resistance and epidemic occurrence. As an exception *Moore* reports two outbreaks due to *Hg* sensitive strains. In Denmark one similar outbreak was recorded. All of the three epidemics occurred in maternity wards. The lack of correlation between the *Hg* reaction and mortality, production of alpha lysin and hyaluronidase and the *IY* reaction does not mean that the *Hg* reaction may not reflect an important metabolic or structural property of the strains.

The *phage sensitivity* of the strains is an example of a property related to some facets of pathogenicity and not to others. Type 71 strains have been demonstrated to be typical for impetigo lesions and cutaneous boils are most often caused by group I strains but in an established bacteremic condition phage sensitivity cannot be shown to influence the final outcome of the infection. Neither are certain types more likely to cause bacteraemia than others as phage types of the bacteraemia material correspond closely to those of about 7000 strains.

cases of bacteraemia caused by type 83A strains. The cases occurred in 14 different hospitals where there was no probable epidemiological connection. Therefore the almost constant connection between this phage type and resistance to penicillin, streptomycin and tetracyclines, negative *IY* reaction and *Hg* resistance may be a characteristic of the phage-type. The type has been uncommon but is encountered with increasing frequency. It made up 3 per cent of about 7000 strains from 1960 and 16 bacteraemia cases in 1961 were caused by type 83A. Its occurrence will be followed with attention.

#### CONCLUSIONS AND SUMMARY

When bacteraemia has been established the outcome is

Year period

a number of bacterial characters and mortality, and to investigate to what extent mortality rate was influenced by other known factors

The total mortality rate was 39 per cent. Prognosis was greatly influenced by the coexistence of serious primary diseases, and mortality rate was higher in hospital infections than in infections contracted outside hospitals. Infections due to penicillin- and streptomycin-resistant strains have a mortality rate about twice that for infections due to sensitive strains. Bacteraemia due to EY-negative strains is more often fatal than cases due to infection with EY-positive strains. The EY reaction may be of some value in epidemiologic investigations.

Mercury-resistance is not primarily related to mortality but is intimately correlated to resistance to penicillin and streptomycin, and is most common in strains of the phage-types 80/81, 52 52A/80 81 and 83A.

When the influence on mortality of the origin of the infection, the antibiotic resistance of the strains etc. are eliminated, the phage type of the strains is unrelated to mortality. Recently, several serious infections due to phage-type 83A were recorded. It is suggested that the characteristic properties of these strains may favour their future epidemic occurrence.

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# ZUR SEROLOGIE DER SALMONELLA O-GRUPPEN

30, 42, 13, 48 und 50

Von

F KAUFFMANN und A PETERSEN

Eingegangen 30 XI 00

In Fortsetzung einer vorhergehenden Mitteilung von F Kauffmann & A Petersen „Zur Serologie der Salmonella O-Gruppen 28, 40, 45 und 47“ berichten wir im folgenden über Untersuchungen der O-Gruppen 30, 42, 43, 48 und 50. Da auch diese O-Gruppen komplex gebaut sind, so wurde hierauf näher eingegangen, zumal diese Verhältnisse nicht allgemein bekannt sind. Ferner wurden im Laufe der letzten Jahre zahlreiche *species* dieser O-Gruppen gefunden, ohne dass in allen Fällen eine Einordnung in die betreffenden O-Untergruppen vorgenommen wurde. Es sei jedoch betont, dass diese O-Gruppen im Kauffmann-White-Schema nach wie vor mit 30, 42, 43, 48 und 50 bezeichnet werden, sodass also die O-Untergruppen hierin nicht berücksichtigt werden.

## Die O-Gruppe 30

Wie F Kauffmann & A Boure mitteilten, ist das O-Antigen von *S. soerenga* = 30<sub>1</sub> 1, w. von dem O-Antigene des Test-Stammes der O-Gruppe 30 *S. urbana* verschieden. *S. soerenga* war nicht in der Lage, das O-Serum von *S. urbana* völlig zu erschöpfen, da Agglutinine für *S. urbana* im Serum zurückblieben. Andererseits wurde aber das O-Serum von *S. soerenga* durch *S. urbana* restlos erschöpft. Die gegenwärtigen Antigenbeziehungen können daher folgendermassen ausgedrückt werden:

*S. urbana* = 30<sub>1</sub>, 30<sub>2</sub>

*S. soerenga* = 30<sub>1</sub>

Durch Absorption des O-Serum von *S. urbana* mit *S. soerenga* wurde das 30-Faktor-Serum hergestellt und eine Untersuchung aller bekannten *species* der O-Gruppe 30 hierin vorgenommen. Es zeigte sich:

*S. urbana* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100

ii number of bacterial characters and mortality, and to investigate to what extent mortality rate was influenced by other known factors

The total mortality rate was 39 per cent. Prognosis was greatly influenced by the coexistence of serious primary diseases, and mortality rate was higher in hospital infections than in infections contracted outside hospitals. Infections due to penicillin- and streptomycin resistant strains have a mortality rate about twice that for infections due to sensitive strains. Bacteraemia due to LY-negative strains is more often fatal than cases due to infection with LY-positive strains. The LY reaction may be of some value in epidemiological investigations.

Mercury-resistance is not primarily related to mortality but is intimately correlated to resistance to penicillin and streptomycin, and is most common in strains of the phage-types 80/81, 52/52A/80/81 and 83A.

When the influence on mortality of the origin of the infection, the antibiotic resistance of the strains etc. are eliminated, the phage type of the strains is unrelated to mortality. Recently, several serious infections due to phage-type 83A were recorded. It is suggested that the characteristic properties of these strains may favour their future epidemic occurrence.

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Die O Gruppe 43 kann in 3 O Untergruppen eingeteilt werden

43<sub>1</sub>, 43<sub>2</sub>, 43<sub>3</sub> = *S. milwaukee*

43<sub>1</sub>, 43<sub>2</sub> = *S. lingabwa*, *S. ahua*, *S. berkeley*, *S. farcha*, *S. igrigny*, *S. mbao* und *S. luindorp*

43<sub>1</sub>, 43<sub>3</sub>, 43<sub>4</sub> = *S. bunnik*, *S. houten*, Arizona 21 und *Escherichia* 86 B7

Um zu einer Vereinfachung der Antigenformeln zu gelangen, kann man den Faktor 43<sub>3</sub> fortlassen, sodass man nur 2 verschiedene O Untergruppen unterscheidet

43<sub>1</sub>, 43<sub>2</sub> = *S. ahua*, *S. berkeley*, *S. farcha*, *S. igrigny*, *S. lingabwa*, *S. mbao*, *S. milwaukee* und *S. luindorp*

43<sub>1</sub>, 43<sub>3</sub> = *S. bunnik*, *S. houten*, Arizona 21 und *Escherichia* 86 B7

Mit Hilfe von 2 Faktor-Seren 43 und 43<sub>1</sub> ist es bereits in der Objektglas Agglutination möglich, die Diagnose zu stellen

Kultur	Serum 43	Serum 43 <sub>1</sub>
43 <sub>1</sub> , 43 <sub>2</sub>	++	—
43 <sub>1</sub> , 43 <sub>3</sub>	—	++

In der Tabelle 1 sind einige der ausgeführten Absorptions-Versuche, aus denen die obigen Formeln abgeleitet wurden, wiedergegeben

Die zur Differentialdiagnose der O Untergruppen nötigen Faktor-Seren können wie folgt hergestellt werden

43-Serum = O Serum von *S. milwaukee* absorbiert mit Arizona 21

43<sub>1</sub> Serum = O Serum von Arizona 21 absorbiert mit *S. milwaukee*  
Anstelle von Arizona 21 kann man auch *S. bunnik* oder *S. houten* benutzen

Weitere, nicht in der Tabelle 1 angegebene Absorptionsversuche zeigten, dass das Arizona 21 Serum von *S. bunnik* oder *S. houten* völlig erschöpft wurde. Ferner wurde durch gekreuzte Absorption die O Identität von *S. bunnik* und *S. houten* festgestellt

Wie schon seit langem bekannt, sind Arizona O 21 und *E. coli* 86 hinsichtlich ihrer O Antigene sehr nahe verwandt, doch nicht völlig identisch. Praktisch gesehen, kann man sie aber als identisch betrachten und zur O Untergruppe 43<sub>1</sub>, 43<sub>2</sub>, 43<sub>3</sub> rechnen

Im originalen Kauffmann White Schema werden aber nach wie vor alle Mitglieder der O 43 Gruppe nur mit 43 bezeichnet und durch ein polyvalentes Serum, das mit *S. milwaukee* + *S. bunnik* oder Arizona 21 hergestellt ist, diagnostiziert

#### Die O Gruppe 48

Diese O Gruppe kann in 2 O Untergruppen eingeteilt werden

48<sub>1</sub>, 48<sub>2</sub> = *S. dahlum*, *S. hammonia*, *S. ngozi* und *S. sakaraha*

48<sub>1</sub>, 48<sub>2</sub>, 48<sub>3</sub> = *S. djakarta* und *S. husingen*

Zu dieser O Gruppe 48 gehören auch die *S. arizonae* species 5 17 20 = 48<sub>3</sub> sowie eine neue von Dr. Cohen, Sofia, erhaltene *S.*



*S. angoda*, *S. aqua*, *S. bodjonegoro*, *S. donna*, *S. gege*, *S. kumasi*, *S. landau*, *S. matopeni*, *S. morehead*, *S. morocco*, *S. odozi*, *S. ramat-gan* und *S. zehlendorf*.

### Die O-Gruppe 42

In der entsprechenden Weise wie in der O Gruppe 30 war auch in der O-Gruppe 42 von *F. Kauffmann & K. Bore* festgestellt worden, dass  $S. loenga = 1,42 \cdot z_{10} z_6$  hinsichtlich des O-Antigenes von dem Teststamme dieser Gruppe *S. weslaco* verschieden war. Während das O-Serum von *S. weslaco* durch *S. loenga* völlig erschöpft wurde, konnten  $S. weslaco + S. senftenberg$  das O-Serum von *S. loenga* nicht restlos absorbieren. Es blieben Agglutinine für *S. loenga* im Serum zurück. Die gegenseitigen Antigenbeziehungen können daher folgendermassen ausgedrückt werden.

$$S. weslaco = 42_1$$

$$S. loenga = 1,42_1, 42_2$$

Durch Absorption des O-Serum von *S. loenga* mit  $S. weslaco + S. senftenberg$  wurde das  $42_2$  Faktor-Serum hergestellt und eine Prüfung aller bekannten *species* der O-Gruppe 42 hierin ausgeführt. Es zeigte sich, dass folgende *species* das  $42_2$ -Antigen besaßen: *S. equisito*, *S. harvesthude*, *S. lampala*, *S. kaneshie* und *S. maricopa*. Da alle diese *species* das O 1-Antigen enthalten, so ist dieser Faktor  $42_2$  wahrscheinlich irgendwie an das O 1-Antigen gekoppelt, doch wurde *S. paratyphi A* nicht vom  $42_2$ -Serum agglutiniert. Ebenso wie aber das O 1-Antigen den O 1-Formenwechsel aufweist, liegt auch ein  $42_2$ -Formenwechsel vor. Alle Kolonien, die das O 1-Antigen stark entwickelt haben, besitzen auch ein starkes  $42_2$ -Antigen, während umgekehrt alle Kolonien, welche das O 1-Antigen nur sehr schwach entwickelt haben, auch nur sehr wenig  $42_2$ -Antigen enthalten.

Die folgenden *species* besaßen weder das O 1-Antigen noch den  $42_2$ -Faktor: *S. chinomum*, *S. detroit*, *S. fremantle*, *S. nairobi*, *S. portbeck*, *S. rand* und *S. uphull*. Sie enthielten also nur das  $42_1$ -Antigen.

### Die O-Gruppe 43

Im Jahre 1954 teilten *F. Kauffmann & J. Vandepitte* mit, dass eine neue *Salmonella species S. kingabwa* ein von *S. milwaukee*, dem Teststamme der O-Gruppe 43, abweichendes O-Antigen habe. Das O-Serum von *S. milwaukee* konnte nicht durch *S. kingabwa* völlig absorbiert werden, es blieb ein Sonderfaktor für *S. milwaukee* im Serum zurück.

Da dieser Sonderfaktor bisher nur bei *S. milwaukee* nachgewiesen wurde, so haben wir jetzt untersucht, bei welchen Kulturen der O-Gruppe 43 dieser Faktor vorkommt. Wie es aus folgender Aufstellung hervorgeht, kommt er auch bei *S. bunnik*, *S. houten*, Arizona O 21 und *Escherichia* 86 B7 vor.

Die O Gruppe 43 kann in 3 O Untergruppen eingeteilt werden

43<sub>1</sub>, 43<sub>2</sub>, 43<sub>3</sub> = *S milwaukee*

43<sub>1</sub>, 43<sub>2</sub> = *S kingabwa*, *S ahuza*, *S berkeley*, *S farcha*, *S irigny*, *S mbao* und *S luindorp*

43<sub>1</sub>, 43<sub>1</sub>, 43<sub>1</sub> = *S bunnik*, *S houten*, Arizona 21 und *Escherichia* 86 B7

Um zu einer Vereinfachung der Antigenformeln zu gelangen, kann man den Faktor 43<sub>3</sub> fortlassen, sodass man nur 2 verschiedene O Untergruppen unterscheidet

43<sub>1</sub>, 43<sub>2</sub> = *S ahu-a*, *S berkeley*, *S farcha*, *S irigny*, *S kingabwa*, *S mbao*, *S milwaukee* und *S luindorp*

43<sub>1</sub>, 43<sub>1</sub> = *S bunnik*, *S houten*, Arizona 21 und *Escherichia* 86 B7

Mit Hilfe von 2 Faktor Seren 43<sub>2</sub> und 43<sub>1</sub> ist es bereits in der Objektglas Agglutination möglich die Diagnose zu stellen

kultur	Serum 43 <sub>2</sub>	Serum 43 <sub>1</sub>
43 <sub>1</sub> 43 <sub>2</sub>	++	—
43 <sub>1</sub> 43 <sub>1</sub>	—	++

In der Tabelle 1 sind einige der ausgeführten Absorptions Versuche, aus denen die obigen Formeln abgeleitet wurden, wiedergegeben

Die zur Differentialdiagnose der O Untergruppen nötigen Faktor Seren können wie folgt hergestellt werden

43<sub>2</sub>-Serum = O Serum von *S milwaukee* absorbiert mit Arizona 21

43<sub>1</sub> Serum = O Serum von Arizona 21 absorbiert mit *S milwaukee*  
Anstelle von Arizona 21 kann man auch *S bunnik* oder *S houten* benutzen

Weitere, nicht in der Tabelle 1 angegebene Absorptionsversuche zeigten, dass das Arizona 21 Serum von *S bunnik* oder *S houten* völlig erschöpft wurde. Ferner wurde durch gekreuzte Absorption die O Identität von *S bunnik* und *S houten* festgestellt

Wie schon seit langem bekannt, sind Arizona O 21 und *E coli* 86 hinsichtlich ihrer O Antigene sehr nahe verwandt, doch nicht völlig identisch. Praktisch gesehen, kann man sie aber als identisch betrachten und zur O Untergruppe 43<sub>1</sub>, 43<sub>2</sub>, 43<sub>1</sub> rechnen

Im originalen *Kauffmann W hite-Schema* werden aber nach wie vor alle Mitglieder der O 43 Gruppe nur mit 43 bezeichnet und durch ein polyvalentes Serum, das mit *S milwaukee* + *S bunnik* oder Arizona 21 hergestellt ist, diagnostiziert

### Die O Gruppe 48

Diese O Gruppe kann in 2 O Untergruppen eingeteilt werden

48<sub>1</sub>, 48<sub>2</sub> = *S dahlum*, *S hammonia*, *S ngozi* und *S sakaraha*

48<sub>1</sub>, 48<sub>2</sub>, 48<sub>3</sub> = *S djakarta* und *S husingen*

Ferner gehören zu dieser O Gruppe 48 die *S arizonae-species* 5 17,20 = 48 <sub>234</sub> - sowie eine neue von Dr Cohen, Sofia, erhaltene *S*

TABELLE 1

O Gruppe 43

## S milwaukee O Serum

kultur	Nicht absorbiert	Absorbiert mit	
		S kingabwa	Arizona 21
S milwaukee	512	256	256
S kingabwa	512	0	256
Arizona 21	64	8	0
E coli 86 B7	64	16	0
Faktoren	43 <sub>1</sub> 43 <sub>2</sub> 43 <sub>3</sub>	43 <sub>3</sub>	43

## S kingabwa O Serum

kultur	Nicht absorbiert	Absorbiert mit	
		S milwaukee	Arizona 21
S milwaukee	512	0	256
S kingabwa	512	0	512
Arizona 21	64	0	0
E coli 86 B7	32	8	0
Faktoren	43 <sub>1</sub> 43		43 <sub>2</sub>

## Arizona O 21 Serum

kultur	Nicht absorbiert	Absorbiert mit	
		S milwaukee	S kingabwa
S milwaukee	512	0	128
S kingabwa	512	0	0
Arizona 21	512	128	128
E coli 86 B7	256	128	128
Faktoren	43 <sub>1</sub> 43 <sub>2</sub> 43 <sub>3</sub>	43 <sub>1</sub>	43 <sub>2</sub> 43 <sub>3</sub>

0 = negativ in 1-4

arizonae species = 48 k c n die ein abweichendes O Antigen besitzt. Der Test Stamm der Arizona O 5-Gruppe (= Sal 48 z<sub>3</sub>) hat die O Antigene 48<sub>1</sub>, 48<sub>2</sub>, 48<sub>3</sub> und ist hinsichtlich seiner O Antigene mit *S. dysenteriae* identisch. Die neue species 48 k c n wurde bisher noch nicht näher untersucht.

Schliesslich gehören zur O 48 Gruppe die atypischen *Citrobacter* Stämme 2624/36 und 5396/38 = Sal 48, V<sub>1</sub> - 1,5, welche die O Antigene 48<sub>1</sub>, 48<sub>2</sub>, 48<sub>3</sub> enthalten.

Bei diesen atypischen *Citrobacter*-Stämmen handelt es sich um Kulturen, die früher mit „*Salmonella coli*“ bezeichnet wurden. Sie nehmen tatsächlich eine Zwischenstellung zwischen *Salmonella*, *E. coli* und *Citrobacter* ein und sind wahrscheinlich Hybriden. Da sie biochemisch am nächsten dem *genus Citrobacter* stehen, obwohl sie Indol bilden und sich hierin wie *E. coli* verhalten, so haben Erber & Kauffmann sie atypische *Citrobacter*-Stämme genannt. Sie sind nämlich KCN-positiv, bilden H<sub>2</sub>S und sind in Ammonium Citrat positiv. Von *Salmonella* unterscheiden sie sich durch den positiven KCN-Test, die Indol-Bildung und die Lactose-Spaltung. Sie stehen serologisch dem *genus Salmonella* sehr nahe, da sie zur O-Gruppe 48 gehören, das Vi-Antigen und die H-Antigene 1,5 besitzen.

Das O-Antigen dieser Kulturen war früher mit 31 bezeichnet, wurde aber später aus dem *Salmonella* Schema ausgeschlossen. Erber & Kauffmann wiesen nach, dass *S. djakarta* = 31 z<sub>1</sub>, z<sub>2</sub> - und *Citrobacter* 2624 36 nahe verwandte O-Antigene besitzen.

TABELLE 2  
O-Gruppe 48

Kultur	O-Seren			
	S. dahlum		S. djakarta	
	Nicht absorb.	Absorb. mit Citr. 2624 36	Nicht absorb.	Absorb. mit S. dahlum
S. dahlum	640	512	320	0
S. djakarta	1280	1024	1280	512
Ar 48 z <sub>30</sub>	80	128	320	128
Citr. 2624 36	10	0	80	64
Faktoren	48 <sub>1</sub> 48 <sub>2</sub>	48 <sub>3</sub>	48 <sub>1</sub> 48 <sub>2</sub> 48 <sub>3</sub>	48 <sub>3</sub>

Citrobacter 2624 36

Kultur	Nicht absorb.	Absorbiert mit		
		S. djakarta	S. dahlum	S. dahlum + Ar 48
S. dahlum	20	0	0	0
S. djakarta	640	0	1024	0
Ar 48 z <sub>30</sub>	40	0	128	0
Citr. 2624 36	640	256	512	256
Faktoren	48 <sub>1</sub> 48 <sub>2</sub> 48 <sub>3</sub>	48 <sub>1</sub>	48 <sub>2</sub> 48 <sub>3</sub>	48 <sub>1</sub>

Die nicht absorbierten Seren wurden ab 1:10 angesetzt, die absorbierten Seren ab 1:4, da sie in der Verdünnung 1:2 absorbiert wurden. 0 = negativ in 1:4.  
Die Identität von S. djakarta und Arizona 48 z<sub>30</sub> wurde durch gekreuzte Absorptionsversuche nachgewiesen.

Im Supplement II zum *Kauffmann-White-Schema* hat *Kauffmann* mitgeteilt, dass die O-Gruppen 31 und 48 zu einer einzigen O Gruppe 48 vereint wurden, da *S djakarta* – wie *J Taylor* nachwies – mit *S dahlem* nahe verwandt ist. Die O-Gruppe 31 wurde gestrichen, da *S djakarta* nicht vollständig glatt war.

Der Test-Stamm der O-Gruppe 48, *S dahlem*, wurde von *Kauffmann, Hofmann & Platz* beschrieben. Die Autoren teilten mit, dass das O Serum von *S dahlem* durch Arizona O 5 völlig absorbiert wurde, dass aber *S dahlem* nicht imstande sei, das O-Serum von Arizona O 5 zu erschöpfen.

Die O-Antigenbeziehungen zwischen *Salmonella* 48, Arizona 5 und den atypischen *Citrobacter*-Stämmen waren also schon bekannt und sind in dieser Arbeit nur näher analysiert worden.

Die verschiedenen Kulturen verteilen sich auf die einzelnen O Untergruppen wie folgt.

48<sub>1</sub>, 48<sub>2</sub> = *S dahlem*, *S hammonia*, *S ngozi* und *S sakaraha*

48<sub>1</sub>, 48<sub>2</sub>, 48<sub>3</sub> = *S djakarta*, *S husing-n* und Arizona 48

48<sub>1</sub>, 48<sub>3</sub>, 48<sub>4</sub> = *Citrobacter* 2624/36 und 5396/38

Zur Differentialdiagnose gebraucht man also 3 Faktor-Sera

48<sub>2</sub> = O-Serum von *S dahlem* oder von *S djakarta* absorbiert mit *Citrobacter* 2624/36

48<sub>3</sub> = O-Serum von *S djakarta* oder von Arizona 48 absorbiert mit *S dahlem*

48<sub>4</sub> = O-Serum von *Citrobacter* 2624/36 absorbiert mit *S djakarta* oder Arizona 48

Die hier angegebenen O-Partialantigene ergeben sich aus verschiedenen Absorptions-Versuchen, deren Resultate in der Tabelle 2 angegeben sind.

Es geht aus der Tabelle 2 hervor, dass die Bestimmung der O-Untergruppen mit Hilfe von 3 Faktor-Seren 48<sub>2</sub>, 48<sub>3</sub> und 48<sub>4</sub> vorgenommen werden kann, und zwar mit Hilfe der Objektglas-Agglutination.

Kultur	48	48 <sub>2</sub>	48 <sub>4</sub>
<i>S dahlem</i>	++	—	—
<i>S djakarta</i>	++	++	—
Arizona 48	++	++	—
<i>Citrobacter</i> 2624/36	—	++	++

Zur Diagnose der verschiedenen *Salmonella-species* der O Gruppe 48 wird empfohlen, ein polyvalentes O Serum von *S dahlem* + *S djakarta* oder Arizona 48 zu benutzen.

Lässt man die *Citrobacter* Kulturen ausser Betracht, so kann man die beiden Untergruppen vereinfacht mit 48<sub>1</sub> = *S dahlem* und 48<sub>1</sub>, 48<sub>3</sub> = *S djakarta* angeben, gebraucht also zur Diagnose nur ein einziges Faktor-Serum 48<sub>3</sub> (*S djakarta* O-Serum absorbiert mit *S dahlem*).

## Die O Gruppe 50

Zu dieser O Gruppe gehören bisher folgende O Untergruppen

50<sub>1</sub> 50<sub>2</sub>, 50<sub>4</sub> = *S greenside* und *S krugersdorp*

50<sub>1</sub>, 50<sub>2</sub>, 50<sub>3</sub> = *S bonaire* und *S wassenaar*

50<sub>1</sub>, 50<sub>3</sub> = *S hooggraven*

Ferner gehören hieher die *Arizona species*

9a 9b = 50<sub>1</sub>, 50<sub>2</sub>, 50<sub>3</sub> (= 50 G -)

9a, 9c = 50<sub>1</sub>, 50<sub>2</sub>, 50<sub>4</sub> (= 50 1 z)

Die O Identität von *S arizonae* 9a 9c mit *S greenside* war bereits in der Arbeit von Schrite, Kauffmann & Edwards mitgeteilt worden

TABELLE 3

O Gruppe 50

Kultur	O-Seren				
	S greenside			S hooggraven	
	Nicht absorb	Absorbiert mit		Nicht absorb	Absorb mit S greenside
		S hoog graven	F 3a 3b		
S greenside	1280	512	128	160	0
S hooggraven	80	0	0	1280	512
Ar 9a 9b	1280	512	0	1280	512
Ar 9a 9c	320	256	16	160	0
F coli 55 115	2560	1024	0	320	0
Faktoren	50 <sub>1</sub> 50 <sub>2</sub> 50 <sub>4</sub>	50 50 <sub>3</sub>	(50 <sub>4</sub> )	50 <sub>1</sub> 50 <sub>3</sub>	50 <sub>3</sub>

Das II Serum von S hooggraven wurde durch Arizona 9a 9b völlig absorbiert

Kultur	O-Seren				
	Arizona 9a 9b			Arizona 9a 9c	
	Nicht absorb	Absorbiert mit		Nicht absorb	Absorb mit Ar 9a 9b
		Ar 9a 9c	S hoog graven		
S greenside	320	0	128	320	84
S hooggraven	320	128	0	320	0
Ar 9a 9b	640	128	256	640	8
Ar 9a 9c	160	0	32	320	64
F coli 55 115	640	0	256	1280	128
Faktoren	50 <sub>1</sub> 50 50 <sub>2</sub>	50 <sub>3</sub>	50	50 <sub>1</sub> 50 <sub>2</sub> 50 <sub>4</sub>	50 <sub>1</sub>

Die O-Identität von „Arizona“ 9a, 9b mit *S wassenaar* wurde erst jetzt von uns festgestellt. Es besteht jedoch nicht nur O-Identität zwischen diesen beiden *species*, sondern diese *species* enthalten auch dieselben H-Antigene und gehören zu demselben Biotyp. Es handelt sich also um ein und dieselbe *species*, die zum *sub-genus* II gerechnet wird. Es sei aber betont, dass es sich hierbei um einen stark abweichenden Biotyp des *sub-genus* II handelt, da die Reaktionen in Dulcitol, Mukat und Malonat negativ sind, während der KCN-Test positiv ist und Schiem nach 1-2 Tagen gespalten wird.

Ferner gehört zur O-Gruppe 50 — wie seit langem bekannt — die *Escherichia-species* 55. B5 6, deren O-Antigene mit 50<sub>1</sub>, 50<sub>2</sub>, (50<sub>3</sub>) angegeben werden können.

Zwecks Vereinfachung kann man den 50<sub>1</sub>-Faktor auslassen, sodass die Antigenstruktur wie folgt angegeben werden kann:

50<sub>1</sub>, 50<sub>2</sub> = *S greenside*, *S krugersdorp*, Arizona 9a, 9c und *Escherichia* 55. B5

50<sub>1</sub>, 50<sub>2</sub>, 50<sub>3</sub> = *S bonaire* und *S wassenaar*

50<sub>1</sub>, 50<sub>3</sub> = *S hooggraven*

Die „Arizona“-Kultur 9a, 9b wurde aus dem obigen Schema ausgeschlossen, da sie mit *S wassenaar* identisch ist. Zur Differentialdiagnose dieser 3 O-Untergruppen benötigt man also nur 2 Faktor-Seren 50<sub>2</sub> und 50<sub>3</sub>.

Diese Befunde stimmen mit den Angaben von *Guinee*, *Kampelmacher* & *Willems*, die *S wassenaar* und *S bonaire* beschrieben, überein. Die Autoren fanden, dass *S wassenaar* und *S bonaire* sich gleich verhielten und von *S greenside* verschieden waren. Es wurden aber keine Versuche, aus denen die O-Identität von *S wassenaar* und *S bonaire* hervorging, mitgeteilt.

Die hier angeführten O-Partialantigene der O-Gruppe 50 ergeben sich aus zahlreichen Absorptions-Versuchen, die zum Teil in Tabelle 3 wiedergegeben sind.

Es geht aus der Tabelle 3 hervor, dass die Bestimmung der O-Untergruppen mit Hilfe von 3 Faktor-Seren 50<sub>2</sub>, 50<sub>1</sub> und 50<sub>3</sub> vorgenommen werden kann, und zwar mit Hilfe der Objektglas-Agglutination.

Kultur	50 <sub>2</sub>	50 <sub>1</sub>	50 <sub>3</sub>
<i>S greenside</i>	++	—	++
<i>S hooggraven</i>	—	++	—
<i>S wassenaar</i>	++	++	—

Faktor-Serum 50<sub>2</sub> — *S wassenaar* O-Serum absorbiert mit *S hooggraven*

Faktor-Serum 50<sub>3</sub> = *S hooggraven* O-Serum absorbiert mit *S greenside*

Faktor-Serum 50<sub>1</sub> = *S greenside* O-Serum absorbiert mit *S wassenaar* oder Arizona 9a, 9c O-Serum absorbiert mit Arizona 9a, 9b

Wie es aus Tabelle II hervorgeht, sind *S. greenside* und *E. coli* 55 B5 nicht völlig identisch, da das O-Serum von *E. coli* 55 B5 zwar durch *S. greenside* völlig absorbiert wird, aber umgekehrt das O-Serum von *S. greenside* nicht durch *E. coli* 55 B5 erschöpft wird. Um diese Differenz zum Ausdruck zu bringen, wurde die Formel von *E. coli* 55. B5 mit 50<sub>1</sub>, 50<sub>2</sub>, (50<sub>3</sub>) angegeben.

Will man – wie bereits erwähnt – die Diagnose der 3 O-Untergruppen vereinfachen, so kann man den 50<sub>1</sub>-Faktor ausser Betracht lassen und die Differentialdiagnose nur mit Hilfe der 50<sub>2</sub>- und 50<sub>3</sub>-Faktoren stellen.

*S. greenside* + *S. krugersdorp* = 50<sub>1</sub>, 50<sub>2</sub>

*S. wassenaar* + *S. bonaire* = 50<sub>1</sub>, 50<sub>2</sub>, 50<sub>3</sub>

*S. haaggraven* = 50<sub>1</sub>, 50<sub>3</sub>

Zur Diagnose der verschiedenen *Salmonella* species der O Gruppe 50 wird empfohlen, ein polyvalentes O-Serum von *S. greenside* + *S. wassenaar* zu benutzen, damit alle 4 Partialfaktoren 50<sub>1</sub>, 50<sub>2</sub>, 50<sub>3</sub> und 50<sub>4</sub> in diesem Serum vertreten sind.

Betreffs der Antigenbeziehungen zwischen *Salmonella* O 50 und *Arizona* O 9 sei betont, dass die O-Antigene von *S. greenside* = 50<sub>1</sub>, 50<sub>2</sub>, 50<sub>4</sub> mit *S. arizonae* 9a, 9c und dass *S. wassenaar* = 50<sub>1</sub>, 50<sub>2</sub>, 50<sub>3</sub> mit „*Arizona*“ 9a, 9b identisch sind. Der in der Arbeit von Westphal, Kauffmann, Inderitz & Sierlin untersuchte *Arizona* Stamm N 99 der O Gruppe 9 hat die Antigenstruktur 9a, 9b und ist deshalb nicht mit *S. greenside* identisch. Dementsprechend ergab auch die qualitative Bausteinanalyse der Polysaccharide Unterschiede zwischen *S. greenside* und *Arizona* N 99 (= 99). Es sei bei dieser Gelegenheit noch darauf hingewiesen, dass bei einer wiederholten Untersuchung des *Arizona* N 99-Stammes Fucose nicht nachgewiesen werden konnte (siehe bei Kauffmann, Jann Kruger, Inderitz & Westphal). Der einzige Unterschied zwischen *S. greenside* und *Arizona* N 99 besteht also in dem Fehlen von

„*Arizona*“ 9a, 9b andererseits zu denselben Chemo Typen gehören werden.

## ZUSAMMENFASSUNG

Auf Grund einer näheren Untersuchung aller bisher bekannten „*Arizona*“ 9a, 9b, 9c, 9d, 9e, 9f, 9g, 9h, 9i, 9j, 9k, 9l, 9m, 9n, 9o, 9p, 9q, 9r, 9s, 9t, 9u, 9v, 9w, 9x, 9y, 9z, 9aa, 9ab, 9ac, 9ad, 9ae, 9af, 9ag, 9ah, 9ai, 9aj, 9ak, 9al, 9am, 9an, 9ao, 9ap, 9aq, 9ar, 9as, 9at, 9au, 9av, 9aw, 9ax, 9ay, 9az, 9ba, 9bb, 9bc, 9bd, 9be, 9bf, 9bg, 9bh, 9bi, 9bj, 9bk, 9bl, 9bm, 9bn, 9bo, 9bp, 9bq, 9br, 9bs, 9bt, 9bu, 9bv, 9bw, 9bx, 9by, 9bz, 9ca, 9cb, 9cc, 9cd, 9ce, 9cf, 9cg, 9ch, 9ci, 9cj, 9ck, 9cl, 9cm, 9cn, 9co, 9cp, 9cq, 9cr, 9cs, 9ct, 9cu, 9cv, 9cw, 9cx, 9cy, 9cz, 9da, 9db, 9dc, 9dd, 9de, 9df, 9dg, 9dh, 9di, 9dj, 9dk, 9dl, 9dm, 9dn, 9do, 9dp, 9dq, 9dr, 9ds, 9dt, 9du, 9dv, 9dw, 9dx, 9dy, 9dz, 9ea, 9eb, 9ec, 9ed, 9ee, 9ef, 9eg, 9eh, 9ei, 9ej, 9ek, 9el, 9em, 9en, 9eo, 9ep, 9eq, 9er, 9es, 9et, 9eu, 9ev, 9ew, 9ex, 9ey, 9ez, 9fa, 9fb, 9fc, 9fd, 9fe, 9ff, 9fg, 9fh, 9fi, 9fj, 9fk, 9fl, 9fm, 9fn, 9fo, 9fp, 9fq, 9fr, 9fs, 9ft, 9fu, 9fv, 9fw, 9fx, 9fy, 9fz, 9ga, 9gb, 9gc, 9gd, 9ge, 9gf, 9gg, 9gh, 9gi, 9gj, 9gk, 9gl, 9gm, 9gn, 9go, 9gp, 9gq, 9gr, 9gs, 9gt, 9gu, 9gv, 9gw, 9gx, 9gy, 9gz, 9ha, 9hb, 9hc, 9hd, 9he, 9hf, 9hg, 9hh, 9hi, 9hj, 9hk, 9hl, 9hm, 9hn, 9ho, 9hp, 9hq, 9hr, 9hs, 9ht, 9hu, 9hv, 9hw, 9hx, 9hy, 9hz, 9ia, 9ib, 9ic, 9id, 9ie, 9if, 9ig, 9ih, 9ii, 9ij, 9ik, 9il, 9im, 9in, 9io, 9ip, 9iq, 9ir, 9is, 9it, 9iu, 9iv, 9iw, 9ix, 9iy, 9iz, 9ja, 9jb, 9jc, 9jd, 9je, 9jf, 9jg, 9jh, 9ji, 9jj, 9jk, 9jl, 9jm, 9jn, 9jo, 9jp, 9jq, 9jr, 9js, 9jt, 9ju, 9jv, 9jw, 9jx, 9jy, 9jz, 9ka, 9kb, 9kc, 9kd, 9ke, 9kf, 9kg, 9kh, 9ki, 9kj, 9kk, 9kl, 9km, 9kn, 9ko, 9kp, 9kq, 9kr, 9ks, 9kt, 9ku, 9kv, 9kw, 9kx, 9ky, 9kz, 9la, 9lb, 9lc, 9ld, 9le, 9lf, 9lg, 9lh, 9li, 9lj, 9lk, 9ll, 9lm, 9ln, 9lo, 9lp, 9lq, 9lr, 9ls, 9lt, 9lu, 9lv, 9lw, 9lx, 9ly, 9lz, 9ma, 9mb, 9mc, 9md, 9me, 9mf, 9mg, 9mh, 9mi, 9mj, 9mk, 9ml, 9mm, 9mn, 9mo, 9mp, 9mq, 9mr, 9ms, 9mt, 9mu, 9mv, 9mw, 9mx, 9my, 9mz, 9na, 9nb, 9nc, 9nd, 9ne, 9nf, 9ng, 9nh, 9ni, 9nj, 9nk, 9nl, 9nm, 9nn, 9no, 9np, 9nq, 9nr, 9ns, 9nt, 9nu, 9nv, 9nw, 9nx, 9ny, 9nz, 9oa, 9ob, 9oc, 9od, 9oe, 9of, 9og, 9oh, 9oi, 9oj, 9ok, 9ol, 9om, 9on, 9oo, 9op, 9oq, 9or, 9os, 9ot, 9ou, 9ov, 9ow, 9ox, 9oy, 9oz, 9pa, 9pb, 9pc, 9pd, 9pe, 9pf, 9pg, 9ph, 9pi, 9pj, 9pk, 9pl, 9pm, 9pn, 9po, 9pp, 9pq, 9pr, 9ps, 9pt, 9pu, 9pv, 9pw, 9px, 9py, 9pz, 9qa, 9qb, 9qc, 9qd, 9qe, 9qf, 9qg, 9qh, 9qi, 9qj, 9qk, 9ql, 9qm, 9qn, 9qo, 9qp, 9qq, 9qr, 9qs, 9qt, 9qu, 9qv, 9qw, 9qx, 9qy, 9qz, 9ra, 9rb, 9rc, 9rd, 9re, 9rf, 9rg, 9rh, 9ri, 9rj, 9rk, 9rl, 9rm, 9rn, 9ro, 9rp, 9rq, 9rr, 9rs, 9rt, 9ru, 9rv, 9rw, 9rx, 9ry, 9rz, 9sa, 9sb, 9sc, 9sd, 9se, 9sf, 9sg, 9sh, 9si, 9sj, 9sk, 9sl, 9sm, 9sn, 9so, 9sp, 9sq, 9sr, 9ss, 9st, 9su, 9sv, 9sw, 9sx, 9sy, 9sz, 9ta, 9tb, 9tc, 9td, 9te, 9tf, 9tg, 9th, 9ti, 9tj, 9tk, 9tl, 9tm, 9tn, 9to, 9tp, 9tq, 9tr, 9ts, 9tt, 9tu, 9tv, 9tw, 9tx, 9ty, 9tz, 9ua, 9ub, 9uc, 9ud, 9ue, 9uf, 9ug, 9uh, 9ui, 9uj, 9uk, 9ul, 9um, 9un, 9uo, 9up, 9uq, 9ur, 9us, 9ut, 9uu, 9uv, 9uw, 9ux, 9uy, 9uz, 9va, 9vb, 9vc, 9vd, 9ve, 9vf, 9vg, 9vh, 9vi, 9vj, 9vk, 9vl, 9vm, 9vn, 9vo, 9vp, 9vq, 9vr, 9vs, 9vt, 9vu, 9vv, 9vw, 9vx, 9vy, 9vz, 9wa, 9wb, 9wc, 9wd, 9we, 9wf, 9wg, 9wh, 9wi, 9wj, 9wk, 9wl, 9wm, 9wn, 9wo, 9wp, 9wq, 9wr, 9ws, 9wt, 9wu, 9wv, 9ww, 9wx, 9wy, 9wz, 9xa, 9xb, 9xc, 9xd, 9xe, 9xf, 9xg, 9xh, 9xi, 9xj, 9xk, 9xl, 9xm, 9xn, 9xo, 9xp, 9xq, 9xr, 9xs, 9xt, 9xu, 9xv, 9xw, 9xx, 9xy, 9xz, 9ya, 9yb, 9yc, 9yd, 9ye, 9yf, 9yg, 9yh, 9yi, 9yj, 9yk, 9yl, 9ym, 9yn, 9yo, 9yp, 9yq, 9yr, 9ys, 9yt, 9yu, 9yv, 9yw, 9yx, 9yy, 9yz, 9za, 9zb, 9zc, 9zd, 9ze, 9zf, 9zg, 9zh, 9zi, 9zj, 9zk, 9zl, 9zm, 9zn, 9zo, 9zp, 9zq, 9zr, 9zs, 9zt, 9zu, 9zv, 9zw, 9zx, 9zy, 9zz, 9aa, 9ab, 9ac, 9ad, 9ae, 9af, 9ag, 9ah, 9ai, 9aj, 9ak, 9al, 9am, 9an, 9ao, 9ap, 9aq, 9ar, 9as, 9at, 9au, 9av, 9aw, 9ax, 9ay, 9az, 9ba, 9bb, 9bc, 9bd, 9be, 9bf, 9bg, 9bh, 9bi, 9bj, 9bk, 9bl, 9bm, 9bn, 9bo, 9bp, 9bq, 9br, 9bs, 9bt, 9bu, 9bv, 9bw, 9bx, 9by, 9bz, 9ca, 9cb, 9cc, 9cd, 9ce, 9cf, 9cg, 9ch, 9ci, 9cj, 9ck, 9cl, 9cm, 9cn, 9co, 9cp, 9cq, 9cr, 9cs, 9ct, 9cu, 9cv, 9cw, 9cx, 9cy, 9cz, 9da, 9db, 9dc, 9dd, 9de, 9df, 9dg, 9dh, 9di, 9dj, 9dk, 9dl, 9dm, 9dn, 9do, 9dp, 9dq, 9dr, 9ds, 9dt, 9du, 9dv, 9dw, 9dx, 9dy, 9dz, 9ea, 9eb, 9ec, 9ed, 9ee, 9ef, 9eg, 9eh, 9ei, 9ej, 9ek, 9el, 9em, 9en, 9eo, 9ep, 9eq, 9er, 9es, 9et, 9eu, 9ev, 9ew, 9ex, 9ey, 9ez, 9fa, 9fb, 9fc, 9fd, 9fe, 9ff, 9fg, 9fh, 9fi, 9fj, 9fk, 9fl, 9fm, 9fn, 9fo, 9fp, 9fq, 9fr, 9fs, 9ft, 9fu, 9fv, 9fw, 9fx, 9fy, 9fz, 9ga, 9gb, 9gc, 9gd, 9ge, 9gf, 9gg, 9gh, 9gi, 9gj, 9gk, 9gl, 9gm, 9gn, 9go, 9gp, 9gq, 9gr, 9gs, 9gt, 9gu, 9gv, 9gw, 9gx, 9gy, 9gz, 9ha, 9hb, 9hc, 9hd, 9he, 9hf, 9hg, 9hh, 9hi, 9hj, 9hk, 9hl, 9hm, 9hn, 9ho, 9hp, 9hq, 9hr, 9hs, 9ht, 9hu, 9hv, 9hw, 9hx, 9hy, 9hz, 9ia, 9ib, 9ic, 9id, 9ie, 9if, 9ig, 9ih, 9ii, 9ij, 9ik, 9il, 9im, 9in, 9io, 9ip, 9iq, 9ir, 9is, 9it, 9iu, 9iv, 9iw, 9ix, 9iy, 9iz, 9ja, 9jb, 9jc, 9jd, 9je, 9jf, 9jg, 9jh, 9ji, 9jj, 9jk, 9jl, 9jm, 9jn, 9jo, 9jp, 9jq, 9jr, 9js, 9jt, 9ju, 9jv, 9jw, 9jx, 9jy, 9jz, 9ka, 9kb, 9kc, 9kd, 9ke, 9kf, 9kg, 9kh, 9ki, 9kj, 9kk, 9kl, 9km, 9kn, 9ko, 9kp, 9kq, 9kr, 9ks, 9kt, 9ku, 9kv, 9kw, 9kx, 9ky, 9kz, 9la, 9lb, 9lc, 9ld, 9le, 9lf, 9lg, 9lh, 9li, 9lj, 9lk, 9ll, 9lm, 9ln, 9lo, 9lp, 9lq, 9lr, 9ls, 9lt, 9lu, 9lv, 9lw, 9lx, 9ly, 9lz, 9ma, 9mb, 9mc, 9md, 9me, 9mf, 9mg, 9mh, 9mi, 9mj, 9mk, 9ml, 9mm, 9mn, 9mo, 9mp, 9mq, 9mr, 9ms, 9mt, 9mu, 9mv, 9mw, 9mx, 9my, 9mz, 9na, 9nb, 9nc, 9nd, 9ne, 9nf, 9ng, 9nh, 9ni, 9nj, 9nk, 9nl, 9nm, 9nn, 9no, 9np, 9nq, 9nr, 9ns, 9nt, 9nu, 9nv, 9nw, 9nx, 9ny, 9nz, 9oa, 9ob, 9oc, 9od, 9oe, 9of, 9og, 9oh, 9oi, 9oj, 9ok, 9ol, 9om, 9on, 9oo, 9op, 9oq, 9or, 9os, 9ot, 9ou, 9ov, 9ow, 9ox, 9oy, 9oz, 9pa, 9pb, 9pc, 9pd, 9pe, 9pf, 9pg, 9ph, 9pi, 9pj, 9pk, 9pl, 9pm, 9pn, 9po, 9pp, 9pq, 9pr, 9ps, 9pt, 9pu, 9pv, 9pw, 9px, 9py, 9pz, 9qa, 9qb, 9qc, 9qd, 9qe, 9qf, 9qg, 9qh, 9qi, 9qj, 9qk, 9ql, 9qm, 9qn, 9qo, 9qp, 9qq, 9qr, 9qs, 9qt, 9qu, 9qv, 9qw, 9qx, 9qy, 9qz, 9ra, 9rb, 9rc, 9rd, 9re, 9rf, 9rg, 9rh, 9ri, 9rj, 9rk, 9rl, 9rm, 9rn, 9ro, 9rp, 9rq, 9rr, 9rs, 9rt, 9ru, 9rv, 9rw, 9rx, 9ry, 9rz, 9sa, 9sb, 9sc, 9sd, 9se, 9sf, 9sg, 9sh, 9si, 9sj, 9sk, 9sl, 9sm, 9sn, 9so, 9sp, 9sq, 9sr, 9ss, 9st, 9su, 9sv, 9sw, 9sx, 9sy, 9sz, 9ta, 9tb, 9tc, 9td, 9te, 9tf, 9tg, 9th, 9ti, 9tj, 9tk, 9tl, 9tm, 9tn, 9to, 9tp, 9tq, 9tr, 9ts, 9tt, 9tu, 9tv, 9tw, 9tx, 9ty, 9tz, 9ua, 9ub, 9uc, 9ud, 9ue, 9uf, 9ug, 9uh, 9ui, 9uj, 9uk, 9ul, 9um, 9un, 9uo, 9up, 9uq, 9ur, 9us, 9ut, 9uu, 9uv, 9uw, 9ux, 9uy, 9uz, 9va, 9vb, 9vc, 9vd, 9ve, 9vf, 9vg, 9vh, 9vi, 9vj, 9vk, 9vl, 9vm, 9vn, 9vo, 9vp, 9vq, 9vr, 9vs, 9vt, 9vu, 9vv, 9vw, 9vx, 9vy, 9vz, 9wa, 9wb, 9wc, 9wd, 9we, 9wf, 9wg, 9wh, 9wi, 9wj, 9wk, 9wl, 9wm, 9wn, 9wo, 9wp, 9wq, 9wr, 9ws, 9wt, 9wu, 9wv, 9ww, 9wx, 9wy, 9wz, 9xa, 9xb, 9xc, 9xd, 9xe, 9xf, 9xg, 9xh, 9xi, 9xj, 9xk, 9xl, 9xm, 9xn, 9xo, 9xp, 9xq, 9xr, 9xs, 9xt, 9xu, 9xv, 9xw, 9xx, 9xy, 9xz, 9ya, 9yb, 9yc, 9yd, 9ye, 9yf, 9yg, 9yh, 9yi, 9yj, 9yk, 9yl, 9ym, 9yn, 9yo, 9yp, 9yq, 9yr, 9ys, 9yt, 9yu, 9yv, 9yw, 9yx, 9yy, 9yz, 9za, 9zb, 9zc, 9zd, 9ze, 9zf, 9zg, 9zh, 9zi, 9zj, 9zk, 9zl, 9zm, 9zn, 9zo, 9zp, 9zq, 9zr, 9zs, 9zt, 9zu, 9zv, 9zw, 9zx, 9zy, 9zz



Die O-Identität von „Arizona“ 9a, 9b mit *S wassenaar* wurde erst jetzt von uns festgestellt. Es besteht jedoch nicht nur O-Identität zwischen diesen beiden *species*, sondern diese *species* enthalten auch dieselben H-Antigene und gehören zu demselben Biotyp. Es handelt sich also um ein und dieselbe *species*, die zum *sub-genus* II gerechnet wird. Es sei aber betont, dass es sich hierbei um einen stark abweichenden Biotyp des *sub-genus* II handelt, da die Reaktionen in Dulcitol, Mukat und Malonat negativ sind, während der KCN-Test positiv ist und Salicin nach 1–2 Tagen gespalten wird.

Ferner gehört zur O Gruppe 50 – wie seit langem bekannt – die *Escherichia-species* 55 B5 6, deren O-Antigene mit 50<sub>1</sub>, 50<sub>2</sub>, (50<sub>4</sub>) angegeben werden können.

Zwecks Vereinfachung kann man den 50<sub>1</sub>-Faktor auslassen, sodass die Antigenstruktur wie folgt angegeben werden kann:

50<sub>1</sub>, 50<sub>2</sub> = *S greenside*, *S krugersdorp*, Arizona 9a, 9c und *Escherichia* 55 B5

50<sub>1</sub>, 50<sub>2</sub>, 50<sub>3</sub> = *S bonaire* und *S wassenaar*

50<sub>1</sub>, 50<sub>3</sub> = *S hooggraven*

Die „Arizona“-Kultur 9a, 9b wurde aus dem obigen Schema ausgeschlossen, da sie mit *S wassenaar* identisch ist. Zur Differentialdiagnose dieser 3 O-Untergruppen benötigt man also nur 2 Faktor-Seren 50<sub>2</sub> und 50<sub>3</sub>.

Diese Befunde stimmen mit den Angaben von Guinee, Kampelmacher & Willems, die *S wassenaar* und *S bonaire* beschrieben, überein. Die Autoren fanden, dass *S wassenaar* und *S bonaire* sich gleich verhielten und von *S greenside* verschieden waren. Es wurden aber keine Versuche, aus denen die O-Identität von *S wassenaar* und *S bonaire* hervorging, mitgeteilt.

Die hier angeführten O-Partialantigene der O-Gruppe 50 ergeben sich aus zahlreichen Absorptions-Versuchen, die zum Teil in Tabelle 3 wiedergegeben sind.

Es geht aus der Tabelle 3 hervor, dass die Bestimmung der O-Untergruppen mit Hilfe von 3 Faktor-Seren 50<sub>2</sub>, 50<sub>3</sub> und 50<sub>4</sub> vorgenommen werden kann, und zwar mit Hilfe der Objektglas Agglutination.

Kultur	50 <sub>2</sub>	50 <sub>3</sub>	50 <sub>4</sub>
<i>S greenside</i>	++	—	++
<i>S hooggraven</i>	—	++	—
<i>S wassenaar</i>	++	++	—

Faktor Serum 50<sub>2</sub> = *S wassenaar* O-Serum absorbiert mit *S hooggraven*

Faktor-Serum 50<sub>3</sub> = *S hooggraven* O-Serum absorbiert mit *S greenside*

Faktor-Serum 50<sub>4</sub> = *S greenside* O-Serum absorbiert mit *S wassenaar* oder Arizona 9a, 9c O Serum absorbiert mit Arizona 9a, 9b

## ZUR DIFFERENTIALDIAGNOSE DER SALMONELLA-SUB GENERA I, II UND III

Von

F. KAUFFMAN

Eingegangen 8. XI. 62

In einer früheren Mitteilung „Two biochemical sub divisions of the genus *Salmonella*“ hat der Verfasser das genus *Salmonella* in 2 biochemisch definierte sub genera eingeteilt sub genus I (typische *Salmonella* Bakterien) und sub genus II (atypische *Salmonella* Bakterien). Gleichzeitig wies er darauf hin, dass die Arizona Gruppe als *Salmonella* sub genus III bezeichnet werden könne.

Die Differentialdiagnose wurde mit Hilfe von Duleit, des Gelatine-Testes und der organischen Säuren gestellt. Wenn wir nur das typische Verhalten der einzelnen species innerhalb der 3 sub genera berücksichtigen, so können wir ein Schema, das in der folgenden Tabelle wiedergegeben ist, aufstellen.

TABELLE  
Typisches Verhalten der Sub Genera

	I	II	III
Duleit	+1	+1	—
Lactose	—	—	+1 oder x
β Galaktosidase		„der x	+
Gelatine		+	+
d Tartrat	+1	— „der x	oder x
l Weinsäure	d	—	—
l Weinsäure	d	—	—
Natrium Citrat	+1	+2	+2 4
Mukol	+1	+1 „	d
Mak nat		+1 2	+1 2

Zeichenerklärung — negativ (bei Duleit und Lactose nach 30 Tagen) + — positiv +1 — positiv nach 1 Tage x — spät und unregelmässig positiv bei β Galaktosidase nach 1 + Tagen positiv d — differente biochemische Typen

In die Tabelle wurde der β Galaktosidase-Test eingefügt, da auch mit seiner Hilfe Unterschiede zwischen den 3 sub-genera nachweisbar sind. Dieser Test wurde kürzlich von I. Minor & Pen Heng Tsao beschrieben.

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## ZUR DIFFERENTIALDIAGNOSE DER SALMONELLA-SUB GENERA I, II UND III

Von  
F. KALFFMAN

Eingegangen 6. XI. 62

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Typisches Verhalten der Sub Genera

	I	II	III
Dulcitol	+1	+1	—
Lactose	—	—	+1 oder x
β Galaktosidase	—	oder x	+
Gelatine		+	+
d Tartrat	+1	— oder x	— oder x
l Weinsäure	d	—	—
l Weinsäure	d	—	—
Natrium-Citrat	+1	+2	+2 4
Mukal	+1	+1 7	d
Malonat	—	+1 2	+1 2

Zeichenerklärung: — = negativ (bei Dulcitol und Lactose nach 30 Tagen), + = positiv, +1 = positiv nach 1 Tage, x = spät und unregelmässig positiv, bei β Galaktosidase nach 1 4 Tagen positiv, d = differente biochemische Typen.

In die Tabelle wurde der β Galaktosidase Test eingefügt, da auch mit seiner Hilfe Unterschiede zwischen den 3 sub-genera nachweisbar sind. Dieser Test wurde kürzlich von F. Minor & P. ...

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Bei Ablesung nach 1 Tage ergaben also 41 kulturen von 59 positiven kulturen eine positive Reaktion Um hiervon einige Beispiele zu geben seien folgende Resultate angeführt

Sub Genus II	Nach 3 Stunden	1 Tag	4 Tagen
■ <i>detro t</i>	—	—	—
S <i>humber</i>	—	—	—
■ <i>uph II</i>	—	—	—
S <i>stellenbosch</i>	—	—	+
S <i>westpark</i>	—	—	+
S <i>bulawaya</i>	±	+	+
S <i>ch novum</i>	±	+	+
S <i>francoa</i>	±	+	+

Von den 51 kulturen des sub genus III ergaben alle eine positive Reaktion und zwar bereits nach 1-3 Stunden Nur 4 kulturen ergaben nach 3 Stunden eine ± Reaktion waren aber alle nach 1 Tage + Die 6 biochemisch abweichenden kulturen die zum sub genus II gerechnet wurden reagierten alle negativ nach 4 Tagen

Während diese 6 kulturen früher zur Arizona Gruppe gerechnet wurden fasst der Verfasser sie als atypische sub genus II species auf Die biochemischen Abweichungen dieser kulturen sind in folgendem Schema wiedergegeben

	Sal ein	Mukat	Mal nat	KCN	Sorbit
1	+ <sup>2</sup>	—	—	+	+
2	+ <sup>2</sup>	—	—	+	+
3	+ <sup>2</sup>	—	—	+	+
4	+	—	—	+	+
5	+ <sup>2</sup>	—	—	—	+
6	—	+ <sup>2</sup>	—	—	—

Alle 6 kulturen waren in Dulcit und Lactose sowie im  $\beta$  Galaktosidase Test negativ und verflüssigten Gelatine d Tartrat = — oder x 1 und 1 Weinsäure = negativ Natrium Citrat = +<sup>2</sup> doch bei Nr 5 negativ

Das serologische Verhalten dieser kulturen ist in folgender Zusammenstellung gegeben

- 1 — Arizona 396-56 = 10a 10c 13 15 = Sal 40 G
  - 2 — Arizona 1450 53 = 21 1 2 6 = Sal 43 z<sub>1</sub>
  - 3 — Arizona 9959 aa = 92 1 2 5 6 = Sal 21 z<sub>1</sub>
  - 4 — Arizona 99 = 9a 9b 13 15 = Sal 50 G —
  - 5 — Arizona 1675-55 = 39 1 2 6 = Sal C z<sub>1</sub> —
  - 6 — Arizona 142 56 = 11 3, 28 = Sal 43 a c n
- (Näheres siehe bei I Kauffmann & R Rohde)

ONPG	80 mg
aq dest	15 ml
Pufferlösung	5 ml

ONPG = Orthonitrophenyl  $\beta$  D Galaktopyranosid von Light & Co. Colnbrook Bucks, England

Pufferlösung	
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	69 gr
aq dest	40 ml
5n NaOH	75 ml

pH 7.0

aq dest ad	50 ml
------------	-------

Die zu untersuchenden Kulturen wurden auf 1 % Lactoseagar-Platten 24 Std bei 37° C gezüchtet und pro Petrischale in 1 ml NaCl-Lösung aufgeschwemmt. Es wurden dann 0,2 ml Toluol hinzugefügt und  $\frac{1}{2}$  Std bei 37° C im Thermostat bebrütet. Dann wurde zu jedem Glase 1 ml der obigen ONPG-Lösung gegeben, worauf die Röhrchen wieder in den Thermostaten von 37° C kamen. Sie wurden nach 1, 3 und 24 Stunden sowie nach 2, 3 und 4 Tagen abgelesen. Eine starke Gelbfärbung wurde mit +, eine schwache mit  $\pm$  bezeichnet.

Im ganzen wurden 334 Kulturen untersucht.

140 Kulturen des *sub-genus* I, und zwar 111 Kulturen, die Gelatine nicht verflüssigten und 29 Kulturen, die Gelatine verflüssigten, also atypisch waren. Es wurden Vertreter aller O-Gruppen ausgewählt, sofern solche des *sub-genus* I vorhanden waren. Bei den häufigen O-Gruppen B, C, D und E, den klassischen *Salmonella* O-Gruppen, wurden zahlreiche *species* untersucht.

143 Kulturen des *sub-genus* II, und zwar alle bis Ende 1962 bekannten *species*, meist nur 1 Vertreter einer *species*. Hierzu gehören 6 biochemisch atypische Kulturen, die früher zur Arizona-Gruppe gerechnet wurden (siehe weiter unten).

51 Kulturen des *sub-genus* III, die biochemisch typische Arizona-Kulturen waren und Lactose meist prompt oder verzögert spalteten.

Von den 140 Kulturen des *sub-genus* I ergaben 137 Kulturen eine negative Reaktion im  $\beta$ -Galaktosidase-Test nach 4 Tagen. *S. jekelston* = 13,23 + C, n, z, die Gelatine nicht verflüssigte, gab nach 2–4 Tagen eine positive Reaktion, also stark verzögert. Von den 29 Kulturen des *sub-genus* I, welche Gelatine verflüssigten, ergaben 2 *species* eine positive Reaktion im  $\beta$ -Galaktosidase-Test. *S. wayne* war nach 24 Stunden positiv, während *S. ferlac*, die prompt Lactose spaltete, nach 1 Stunde positiv war.

Von den 143 Kulturen des *sub-genus* II ergaben 84 Kulturen eine negative Reaktion, während 59 Kulturen positiv reagierten, und zwar

- 4 Kulturen nach 1–3 Stunden,
- 37 Kulturen nach 1 Tage und
- 18 Kulturen nach 2–4 Tagen

*genus Salmonella* nur biochemisch definieren will, so kommen die 3 *sub genera* als *species* in Betracht. Der Verfasser hält aber dieses nicht für zweckmäßig und hat im Sinne der modernen Klassifikation die *species* als eine Gruppe verwandter, sero-fermentativer Phag-Typen definiert.

#### ZUSAMMENFASSUNG

Es wird über die Differentialdiagnose zwischen den *sub-genera* I, II und III des *genus Salmonella* berichtet, speziell über den  $\beta$ -Galaktosidase Test. Dieser gab beim *sub-genus* I negative, beim *sub-genus* II teils negative, teils verzögert positive und beim *sub-genus* III prompt positive Resultate. Das *sub-genus* II steht also auch in dieser Hinsicht zwischen den *sub-genera* I und III, von denen es aber deutlich verschieden ist.

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Nachdem bereits Nr 4 = Arizona N 99 = Sal 50 G - auf Grund ihrer Identität mit *S. wassenaar* aus der Arizona-Gruppe ausgeschlossen und in das sub-genus II überführt wurde, sollen jetzt auch die übrigen 5 species als sub-genus II-Kulturen betrachtet werden

Auf Grund ihres negativen Verhaltens in Malonat stehen diese atypischen species zwischen den sub-genera I und II, sodass sie also nicht zum sub-genus III gehören. Da wahrscheinlich noch weitere, derartige species vorkommen, so wird es bald zweckmässig sein, diese in einem neuen sub-genus zu vereinen, doch können sie vorläufig als atypische sub-genus II-species betrachtet werden. Dass es sich hier wirklich um eine zwischen sub-genus I und II stehende Gruppe handelt, zeigt das Vorkommen malonat-negativer und gelatine-verflüssigender species innerhalb des sub-genus I, z. B. *S. schleissheim*, *S. abortus bovis* etc.

Die Sonderstellung dieser atypischen sub-genus II-species beruht auf folgenden Merkmalen:

Negatives Verhalten in Dulcitol, Lactose, Mukat und Malonat sowie im  $\beta$ -Galaktosidase-Test

Positives Verhalten in KCN, Salicin und Gelatine

Das typische Verhalten der 3 sub-genera im  $\beta$ -Galaktosidase-Test ist in folgendem Schema wiedergegeben:

Nach	Sub Genera			
	I	II		III
	ca 10%	ca 60%	ca 40%	ca 100%
1 Stunde	—	—	(±)	+
3 Stunden	—	—	±	+
1 Tage	—	—	+	+
4 Tagen	—	—	+	+

Es geht auch aus diesem Schema hervor, dass das sub-genus II eine Zwischenstellung zwischen den sub-genera I und III einnimmt und vom sub-genus III verschieden ist.

Vergleicht man die Ergebnisse des  $\beta$ -Galaktosidase-Testes mit denen des üblichen Lactose-Testes, sieht man, dass die Ergebnisse beim sub-genus I identisch sind. Auch beim sub-genus III stimmen die Resultate prinzipiell überein, da alle hier untersuchten Arizona-Kulturen Lactose prompt oder verzögert spalteten. Mit Hilfe des  $\beta$ -Galaktosidase-Testes kann man die positive Reaktion bereits nach wenigen Stunden ablesen. Beim sub-genus II, das einen negativen Lactose-Test ergibt, fallen die Resultate mit dem  $\beta$ -Galaktosidase-Test verschieden aus, da ca. 60 % der Kulturen negativ und ca. 40 % verzögert positiv reagierten.

Zur Diagnose der 3 sub-genera ist aber dieser Test entbehrlich, da wir mit Hilfe vom Lactose, Dulcitol, Gelatine, d-Tartrat und Malonat die Diagnose stellen können.

Falls man im Sinne der orthodoxen Klassifikation die species des



## STRAINS OF *STAPHYLOCOCCUS AUREUS* WITH INCREASED TOLERANCE TO GENTIAN VIOLET

By

FOLKE NORDBRING

Received 20 VIII 62

The inhibiting effect of gentian violet, even in low concentrations on the growth of *Staphylococcus aureus* has been used in routine bacteriologic work in many laboratories. By adding gentian violet to the blood agar plates, most strains of *Staph. aureus* are completely inhibited. In the laboratory of the Uppsala Department of Bacteriology, gentian violet is added to the sheep blood agar medium in a concentration of approximately 1.3  $\mu$ g of gentian violet per ml of medium.

In this laboratory on the contrary, it has been observed occasionally in recent years that some strains of *Staph. aureus* grow abundantly on the blood agar plates with gentian violet added. These colonies are round and opaque, they are smaller than the normal colonies on blood agar plates and have a faint blue colour. If the strain is haemolytic, a distinct zone of haemolysis surrounds the colony and the staphylococci might well be mistaken for haemolytic streptococci.

Such strains of *Staph. aureus* growing on gentian violet plates have been investigated with regard to their morphologic and other characteristics, resistance to antibiotics and gentian violet, penicillinase production and bacteriophage types: the results are reported here.

### MATERIAL

Twenty-four strains of *Staph. aureus* growing on gentian violet plates (GV strains) were studied. The original specimens sent to the laboratory for examination are listed in Table 1. These strains were derived from 19 patients from several hospitals in different cities. All were resistant to penicillin (benzyl penicillin) according to the paper disk diffusion method of Ericsson, Höglman & Wiclman (6).

Twenty-seven strains of *Staph. aureus* which failed to grow on gentian violet plates ('normal' strains) were investigated at the same time. These strains originated from various sources (listed in Table 1) and were chosen at random among the routine laboratory specimens during the same period as the GV strains (Oct.-Nov. 1959). Twenty-five of the 'normal' strains were resistant to penicillin according to the paper disk method, one strain being isolated from a person who also had a GV strain. Two 'normal' strains were sensitive to penicillin, one being isolated from a person with a simultaneous GV strain. The Oxford strain 209 was used as a control.

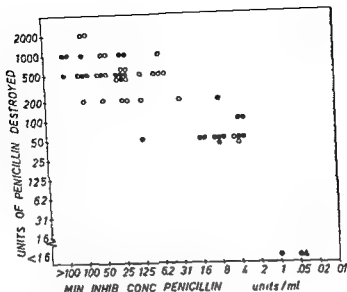


Fig 2

The amount of penicillin destroyed by 0.5 ml of broth culture. Symbols as in Fig 1

tance is more pronounced among the GV strains. The minimal inhibitory concentration of penicillin is 3.1 units per ml or higher for 14 (36 per cent) of the normal strains and for 22 (92 per cent) of the GV strains.

It is seen from the figure that all of the normal strains are inhibited by 0.2–0.4  $\mu$ g of gentian violet per ml except one strain which tolerates 0.8  $\mu$ g per ml. The GV strains tolerate a considerably higher concentration of gentian violet. Most GV strains are inhibited by 1.6–3.1  $\mu$ g per ml (one strain tolerates 8.2  $\mu$ g per ml), i.e. the normal strains are 4–15 times more sensitive to gentian violet than the GV strains.

The amount of penicillin destroyed by 0.5 ml of broth culture of the different strains (approximately 300 million organisms) in one hour of incubation which indirectly represents the penicillinase production is shown in Fig 2. It is readily seen from the figure that there is a fairly good correlation between the extent of penicillin resistance and penicillinase production and there is no difference between GV strains and "normal" strains. No measurable amount of penicillin is destroyed by the three strains susceptible to penicillin. Most strains inhibited by 0.4–1.6 units per ml destroy 50–100 units and most strains requiring 3.1 units per ml or more for inhibition destroy 200–2000 units.

The obvious tendency to pronounced resistance to penicillin among the GV strains raised the question whether these strains were also particularly prone to resist other antibiotics. Table 2 gives the results of the sensitivity tests according to the paper disk method. It is apparent

## RESULTS

All of the strains produced coagulase. Two of the GV strains and two of the "normal" strains were non-haemolytic. There was no difference between the two types of staphylococci as regards the appearance of colonies on blood agar and the pigment production. Most strains exhibited a heavy gold pigmentation. All of the strains fermented sucrose, mannitol, and lactose without production of gas.

The ability of the GV strains to grow on gentian violet agar seemed to be a stable property. The GV strains were kept in the refrigerator on blood agar plates for five months with frequent re-inoculations without losing their increased tolerance to gentian violet. Four GV strains were inoculated on gentian violet plates with seven passages in five weeks without losing their capacity to grow. Thus a continuous contact with gentian violet for several weeks could not provoke a change in tolerance.

The results of the tube tests of susceptibility to penicillin and gentian violet are given in Fig. 1. The two "normal" strains, sensitive to penicillin according to the paper disk method, and the Oxford strain 209 are inhibited by 0.05–0.1 units of penicillin per ml. The remaining strains display a varying degree of resistance to penicillin under the standardized experimental conditions, although the tendency to resist

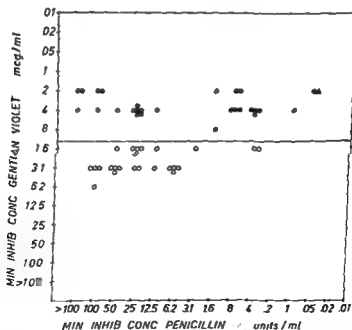


Fig. 1

Results from the serial dilution tests of the sensitivity to penicillin and gentian violet. ● denotes "normal" strains, ○ GV strains, and ▲ the Oxford strain 209. The horizontal line indicates the concentration of gentian violet in the blood agar plates with gentian violet added to the medium.

TABLE 3  
Bacteriophage Patterns of the Strains Studied

No of 1000x RTD	Plaque pattern	Phage group	No of strains	No of nor- mal strains
	1			1
	187			1
	52/80	I		2
	52/80/81	I		4
	80/81/KS6	I		5
1	34/3C	II		3
	3B/3C/54/71	II		1
	53	III	1	
7	54	III	7	
2	6/54	III	2	
	53/54	III	1	
1	54/819	III	1	
1	54/1034	III	2	
	6 47/53	III		1
1	6/53/54	III	1	
1	7/47/54	III	1	
	54/75/77	III		1
1	6 53 54/1034	III	1	
	7/42E/54/819	III		1
1	6/7/47/53/54	III		1
1	6/7/54 819/1034	III	1	
1	6/53 54/77/819	III		1
1	7/42E/54/819/1034	III	1	
1	7/42E/47/54 819 1034	III	1	
1	6 7/53/54/819/1034	III	1	
4	Other patterns	III		5
1	29/42E/53/54	I + III	1	
2	29 42E/53/54/1034	I + III	2	

It is known from the studies by *Park & Strominger* (12, 13, 14) that the inhibiting effect on staphylococci of gentian violet is brought about by its interfering at some point in the reaction sequence leading to cell wall synthesis. This action is similar to that of penicillin, but gentian violet appears to interfere at an earlier stage in the biosynthetic sequence. The mechanism behind the manifold increase in tolerance to gentian violet of the GV strains remains to be elucidated. The resistance to penicillin of the GV strains as expected, was caused by penicillin destruction by penicillinase. Further studies are needed to determine whether GV strains occur exclusively among staphylococci of phage group III.

The capacity to tolerate larger concentrations of gentian violet than normal strains seemed to be a stable property. The capacity did not vanish after several transfers in the laboratory. In two cases the same GV strains were isolated from the same lesion on separate occasions.

from the table that the GV strains have a marked tendency to resistance to various antibiotics and the percentage of resistant GV strains is higher than that of "normal" strains, with the exception of penicillin and the sulphonamides. The resistance of the GV strains to the tetracyclines is particularly marked. It is also notable that 50 per cent of the GV strains are resistant to erythromycin, while only 12 per cent (three strains) of the "normal" ones are resistant to this drug.

The phage patterns of the strains are recorded in Table 3. It is seen from the table that the "normal" strains are scattered throughout the different phage groups, while all of the GV strains fall into phage group III with various patterns. It is noteworthy that a high proportion of the GV strains (22 out of 24) gave lysis only with 1000  $\times$  RTD.

TABLE 2

*Results from the Susceptibility Tests According to the Paper Disk Method  
Two Normal Strains Sensitive to Penicillin Are Excluded*

Agent	Resistant to			
	GV strains		Normal strains	
	No.	%	No.	%
Sulphonamides	21	87	22	89
Penicillin	24	100	25	100
Erythromycin	12	50	3	12
Streptomycin	17	71	8	32
Chlortetracycline	20	83	10	40
Oxytetracycline	21	87	10	40
Tetracycline	20	83	7	28
Chloramphenicol	9	37	3	12
Novobiocin	9	37	3	12

## DISCUSSION

It has been observed by many workers that staphylococci resistant to several antibiotics are almost completely confined to phage group III (1, 3, 4, 9, 11, 15). It has also been particularly pointed out in some studies that the majority of erythromycin resistant staphylococci fall within group III (7, 8, 10). Barber & Whitehead (2) suggested in 1949 that group III staphylococci are genetically less stable than other strains and that resistant mutants develop more readily.

The strains of staphylococci with increased tolerance to gentian violet investigated in the present study were all found to react with bacteriophages of group III and they were all resistant to at least two antibiotics out of the eight tested. Nineteen out of 24 strains (80 per cent) were resistant to five or more antibiotics. This suggests that development of multiple antibiotic resistance may be accompanied by increased resistance to gentian violet.

relapse, the patient had staphylococcal skin boils. Naturally, the bone fistula might have been infected via the skin. The rapid disclosure that the fistula strain grew on gentian violet agar, while the strain from the skin abscesses did not, revealed that the two sites of infection were not epidemiologically connected with each other. This could be stated after overnight incubation, long before the phage-typing was performed.

In fact, the property of tolerance to increased concentration of gentian violet could be used as a means by which to mark staphylococci, together with phage-typing, in epidemiologic studies of various kinds.

## SUMMARY

Strains of staphylococci which grow abundantly on blood agar with gentian violet added (GV strains) have been studied. These strains may be mistaken for beta haemolytic streptococci. They were found to tolerate 4-15 times higher concentrations of gentian violet than other 'normal' strains of staphylococci chosen at random among the routine laboratory specimens. All GV strains studied reacted with bacteriophages of group III and exhibited a marked tendency to multiple resistance to antibiotics.

The GV strains retained their capacity to grow on gentian violet agar after several transfers in the laboratory. A case report is given, demonstrating a long standing infection with a GV strain. It is pointed out that recognition of GV strains may be of value in epidemiologic work.

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The following case history gives a good illustration of a GV strain, which persisted in the tissues for years.

### *Case History*

F.N., a woman 61 years old. In July 1958 supracondylar transverse fracture of the right femur. Operative reposition and fixation of fragments by metal plate and wire was performed. There was a slow but uneventful healing and the fracture seemed to be consolidated one year later. In Sept 1959 signs of infection were noted and a large abscess cavity around the osteosynthesis area was emptied. The osteosynthesis material was removed. A continuously suppurating fistula developed and during the following year the patient was given various antibiotics and sequesters were repeatedly removed. *Staph aureus* was isolated many times from the fistula (phage pattern 1000 X RTD 6/7/47/53/54/75/77, group III) and from the nose and throat (phage type K56/80/81, group I).

In Oct 1960 a first course of methicillin (Belfacillin® Astra) was given. The drug was administered for two weeks and cultures from the fistula secretion were negative after 12 days remaining so for seven weeks although there was still discharge from the fistula. *Staph aureus* was isolated from the fistula in Dec 1960 and again the phage pattern was 1000 X RTD 6/7/47/53/54/75/77. At the same time the patient developed skin abscesses with growth of *Staph aureus* but of another phage pattern K56/80/81.

A second course of methicillin was started in Dec 1960 lasting for seven weeks. The patient was again free from all staphylococci after 12 days and remained so for 10 weeks. There was still a moderate secretion from the fistula. *Staphylococci* of the same phage pattern as previously seen (1000 X RTD 6/47/53/54/75/77) were again isolated from the fistula secretion in March 1961.

A third course of methicillin began in March 1961 and continued for eight weeks. The bacterial cultures from the fistula discharge were negative on the eighth day and the patient was free from staphylococci for 10 weeks. Surgical revision of the osteitis focus was carried out. In June 1961 the same type of *Staph aureus* reappeared in the pus (1000 X RTD 6/7/47/53/54/75/77). A skin furuncle developed some days later but from this focus of infection as before another type of *Staph aureus* was isolated (K56/80/81).

Following two weeks of constant growth of staphylococci of the same type in the fistula secretion a fourth course of methicillin was given for eight weeks from June 1961. The result was as the previous one. The staphylococci disappeared as from the twelfth day but this time the pus remained sterile for six and a half months. During the last two months of this period the patient suffered from several skin boils due to *Staph aureus* 80/81 and this type was frequently cultured from the nose and throat but the fistula secretion remained sterile. However in Jan 1962 the now familiar type of *Staph aureus* (1000 X RTD 6/47/53/54/75/77) was isolated from the fistula.

A fifth course of methicillin followed by oral administration of methyl phenyl isoxazolyl penicillin (Micropenin® Kabi) was started in Febr 1962 with the same quick response as before the cultures being sterile as from the twelfth day.

The staphylococci of group III in the above case, isolated from the bone fistula, grew well on gentian violet plates in Dec 1960 and in Febr 1962 and it is reasonable to believe that the same result would have been obtained earlier, had the strains been investigated in this respect. The staphylococci in this case had obviously been harboured in the bone for years, were capable of re-growth some time after penicillin treatment, and, had retained their property of increased tolerance to gentian violet.

The above case history also illustrates the fact that the recognition of GV strains may be useful epidemiologically. In connection with some of the relapses of the bone infection, or immediately preceding such

## STUDIES OF ANTIBODIES IN HUMAN LISTERIOSIS

### 1 Antibodies in Cases of Bacteriologically Verified Human Listeriosis

By

STEN WINBLAD

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The discovery that *Listeria monocytogenes* (L m) may cause septic disease in animals was made by *Gustav Hulphers* (8) who in 1911 described the first case of such illness in animals. Listeriosis may affect various animals such as sheep rabbits guineapigs fowls horses cattle chinchillas etc. The fact that man too can be thus infected has been known for a long time. Usually the manifestations of such an infection are purulent meningitis or general sepsis in newborn infants or in adults meningitis but also anginous septic and oculo glandular forms have been described. Human listeriosis attracts special interest when occurring pregnancy or in the neonatal period. As a result of *Seeliger's* (19) extensive studies of L m and its infections numerous cases of human listeriosis could be identified in West Germany and recently in other countries too (3 6 7). From Israel has been reported an interesting accumulation of febrile abortions due to infection with L m (*Rappaport et al* 18).

In Denmark *Nyfeldt* (14) claimed to have isolated I m from the blood of patients suffering from infectious mononucleosis during an epidemic in 1929 1930. *Nyfeldt* regarded I m as the cause of this outbreak but this opinion was disputed by many and no later epidemics of this type have been found. In view of our present knowledge however the possibility that *Nyfeldt* was right after all cannot be entirely discarded.

In 1958 the first case in Sweden to be histopathologically and bacteriologically verified was observed (*Finell Schüller & Winblad* 12). A newborn full term child was taken ill when seven days old and died when nine days old. The mother was healthy. Subsequent studies have been carried out during 1960-61.

Table 1 where

10 cases belonged to the prenatal or neonatal period while 10 cases occurred in adults causing meningitis or pleurisy and 20 other cases are associated with the occurrence of bacteria in the placenta or the meconium of healthy children with healthy mothers (*Finell*

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Seeliger & Sulzbacher (21) claim that L m on the one hand has antigens in common with *Staphylococcus aureus* and enterococci on the other. Consequently they consider that an agglutination test for L m must be preceded by absorption of the patient's serum with staphylococci or enterococci in order to be of any diagnostic value. The risk for such overlapping reactions, however, is said to be greater in the agglutination than in the complement fixation test. Further proof that the three bacterial groups have antigens in common is offered by Neler, Anzai & Gorzynski (13) who found cross-reactions between *Staphylococcus aureus* and L m when studied with a passive haemagglutination technique. Welshimer (23) on the other hand, could detect no such common antigens. Experimenting with rabbits he found no L m agglutinins in animals immunized against staphylococci and no staphylococcal agglutinins in those rabbits that had been immunized against L m. At the present moment there are thus divergent opinions concerning the existence of cross reactions between L m and other Gram-positive bacteria.

The object of the present investigation is to study the development of antibodies in human infection with L m using O-agglutinins and complement-fixing antibodies. The first step here will be to examine the antibodies in bacteriologically verified cases, and the second step to study their presence in a larger group of patients with other clinical conditions as well as in healthy subjects. These matters will obviously have to be elucidated before it can be decided whether the occurrence of antibodies in human serum justifies a conclusive diagnosis of listeriosis in those cases where a culture either is not effected or does not yield positive results. The cultivation of L m in cases of clearly established septicaemia or purulent meningitis appears to be fairly simple.

or from women

On such occa-

s desirable

## METHODS

### Antigens for O Agglutination

Listeria m  
rose agar ; " " "  
Dextrose 1 " "  
cent sterile " "  
6.8 This " "  
24 hours " " "  
is harvest " " "  
steamed for 1 hour and then " " "  
and stored at +4° C. " " "  
found suitable for ag " " "  
100 ml of veronal bu " " "  
which is prevented by the heating " " "  
c. 1:1 spontaneous agglutination

<sup>1</sup> kindly supplied by Dr Seeliger Bonn for which I wish to express my gratitude

rell 11) A detailed description of these cases would be beyond the scope of this work, and some of them have been published earlier (1, 2, 4, 5, 9, 10, 12 & 22)

As evidenced by *Seeliger's* investigations, *L. m.* possesses identifiable antigens of the O and H types. *Seeliger's* use of absorbed immune sera from rabbits led to the recognition of five types of the organism 1, 2, 3, 4A and 4B.

TABLE 1  
*Cases of Human Listeriosis in Sweden (Nov 1958-April 1962)*  
*Bacteriologically Verified Cases*

1 *Fetus or newborn children*

	Number	Maturity	Prematurity	Sick mothers	Healthy mothers	*
Meningitis or Septicemia	12	8	4	5	7	-
Abortion	3	-	-	1	2	-
Stillbirth	6	2	4	3	1	2
Asphyxia Dead	2	-	2	2	-	-
Neonocemial infection in maternity hospital	2	2	-	-	2	-
	25	12	10	11	12	2

2 *Adults*

	Number	Healthy	Dead
Meningitis	13	9	4
Pleurisy	1	-	1
Septicemia	1	-	1
	15	9	6

Total cases 40

3 Bacteria in meconium or placenta Healthy children (Laurell)

5

Total bacterial cases 45

Antibodies in man unfortunately provide no conclusive evidence for a satisfactory diagnosis. However, *Seeliger* (19) points out that the complement fixation reaction appears to be more specific than a test for agglutinins in serum where even many healthy subjects may show a comparatively high titer (15, 16, 19, & 21). In the opinion of *Poschel & Schwind* (16) a high agglutination titer should be used for diagnostic purposes only in combination with symptoms indicative of listeriosis.

### Absorptions

Patients sera possessing an agglutination titre of 1/64 or higher have been absorbed by means of the technique described above. In all these cases except one absorption with *Staphylococcus aureus* and enterococci was found to lower the agglutination titre against L m by one step only whereas absorption with the *Listeria* antigen completely removed the anti *Listeria* agglutinins. In one case an unexpected agglutination titre against *Listeria* was found in a child three months old and this titre could be entirely absorbed with enterococci and *Staphylococcus aureus*. It is obvious from the results obtained however, that there are no significant cross reactions between the *Listeria*, *Staphylococcus* and *Enterococcus* antigens employed and also that the antigens used in this investigation with the methods applied should offer reasonable protection against the risk of any cross reactions interfering with specificity.

### *The Development of Antibodies in Mothers of Children with Human Listeriosis*

In 14 cases of listeriosis in newborn infants and abortions it has been possible to follow the development of antibodies in the mothers. These cases showed the following manifestation. One of the newborns exhibited a widespread septicaemia (case 1). In eight cases (cases 2-9) the newborn child had meningitis from which L m could be isolated. Two of the cases (10-11) were abortions where L m was isolated from abortion discharges and the vagina. In other cases (cases 12-13) a premature child was born who died and from whom L m could be recovered. In the last case (case 14) L m was isolated from a stillborn child. Thanks to co-operative colleagues bloodsamples were obtained from the mothers during the period following parturition making it possible to follow the agglutination in all cases and complement fixation titre in 9 cases. The maximal titres thus obtained are recorded in Tables 2 and 3.

In three cases an agglutination titre of merely 32 was found in three others the titre was 64 while higher agglutination titres were found in the rest. Complement fixation tests were performed in 9 cases showing a low titre only once but titres of 1/30 or more in the other nine. Only five studies of antibody titres in healthy subjects (24) prove an agglutination titre of 1/64 or more and a complement fixation titre of 1/30 or more to be titres higher than what is usually found in healthy persons. Thus the titrated titres are obviously elevated more than normally. It is also interesting to note that out of those 14 mothers who are included in the investigation only one was treated with chemotherapy immediately after delivery. There is thus no reason to suspect a chemotherapeutically induced decrease of antigens. The exception is case No. 11 in which early antibacterial treatment was given but in

### *Agglutination Reaction*

Inactivated serum is diluted with saline dichotomically 1/8-1/512 each tube containing 0.25 ml. 0.25 ml of antigen suspension are added, and the tubes are incubated for 18 hours in a water-bath of 52° C and for another 24 hours in a refrigerator. Positive controls with patient serum and rabbit serum are included as well as a negative serum control and a control without serum. Reading by means of a handlens, are made, preferably by observing the appearance of the deposit which often gives a sharper end point than reading after shaking the tubes.

### *Absorption Control*

Sera with a high agglutination titer are re-examined without absorption after absorption with equal amounts of an autoclaved mixture of *Staphylococcus aureus* Cowan's types 1, 2, 3 and 4, with a batch of 7 autoclaved enterococcal strains and with the L m antigen respectively.

### *Antigens for Complement Fixation*

The same method is applied as when antigens for agglutination were prepared except that isotonic saline is used for the suspension. This preparation is boiled for 30 minutes after which its sterility is tested.

### *Complement Fixation Reaction*

Preliminary titrations to determine a suitable dose of the complement fixation antigen are made with a fixed dose of complement and with positive rabbit serum and negative human serum. Further tests to ensure reproducibility of results with different antigen batches, are made with positive and negative human sera. The other steps of the complement fixation test are carried out as usual.

All sera are inactivated at 56° C for 30 minutes.

## RESULTS

If O antigen was prepared in the manner described above, no spontaneous agglutination could be observed on any occasion.

### *H Agglutinins*

Attempts to establish the presence of agglutinins by means of H antigen according to Seeliger were successful in rabbit immunized against according the different strains, but in human sera the results were difficult to read and interpret. As a consequence, the investigation of antibodies against the H-antigen in human sera was abandoned at an early stage.

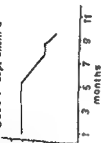
### *Cross-reactions*

Rabbits which had been immunized against the various types of L m showed good production of O agglutinins and complement-fixing antibodies against L m. There was, however, no increase of agglutinins against the mixed antigens of *Staphylococcus aureus* (O antigens from types 2, 3, and 4). Nor did it exhibit an elevated titre against O antigens from the mixed enterococcal strains. It was also found that rabbits immunized with O antigens from the above mentioned types of staphylococci completely lacked agglutinins against *Listeria*. Our findings are thus in perfect agreement with Welshimer & (23) in this respect.

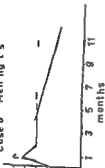
Antibody curves in mothers of newborns - cases of human listeriosis

Comp Agg  
fx 240 512  
120 256  
60 128  
30 64  
15 32  
7 16  
0

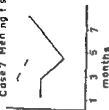
Case 1 Sept chem a



Case 5 Men ng t s

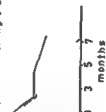


Case 7 Men ng t s

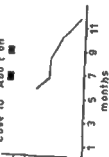


Compl Agg  
fx 240 512  
120 256  
60 128  
30 64  
15 32  
7 16  
0

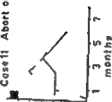
Case 8 Men ng t s



Case 10 Abort on

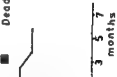


Case 11 Abort on



Compl Agg  
fx 240 512  
120 256  
60 128  
30 64  
15 32  
7 16  
0

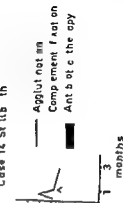
Case 12 P ematur ty Dead



Case 13 Prematur ty Dead



Case 14 St illb th



Agglutination  
Complement fixation  
Antibody titer



spite of this no depression of the antibody curve is to be found. In three cases, however, antibacterial therapy has been administered in the later stages. As seen from Table 3, this investigation shows that an elevated antibody titre, as measured with this technique, is present in agglutination tests in 11 cases out of 14, in complement fixation tests in 8 cases out of 9, and if both antibodies are taken together, in 8 cases out of 9.

TABLE 2

*Maximal Antibody Titre in Cases with Bacteriologically Verified Human Listeriosis*

Case	Disease	Maximal titre in convalescent periods		Antibiotic therapy	
		Agglutination	Complement fix.	Initially	Late
1	Mother of newborn with septicæmia	256	—	—	—
2	Mother of newborn with meningitis	32	—	—	—
3	"	32	—	—	—
4	"	32	—	—	—
5	"	64	160	—	—
6	"	256	240	—	—
7	"	256	240	—	—
8	"	512	120	—	—
9	"	64	10	—	—
10	Mother with abortion	128	—	—	+
11	"	128	120	+	—
12	Mother of premature child Dead	512	120	—	+
13	"	64	60	—	+
14	"	256	40	—	—
15	Adult Meningitis	128	30	+	—
16	"	96	240	+	—
17	"	128	0	+	—
18	"	256	20	+	—
19	"	8	40	+	—
20	"	32	30	+	—

TABLE 3

*Frequency of Elevated Titre of Antibodies in Cases of Human Listeriosis*

Patients	Number	Agglutination		Number	Complement fixation		Elevated titre
		+	-		%	30	Agglutination
Mothers of newborn with listeriosis	14	11	3	9	8	1	8
Adults Meningitis	6	4	2	5	4	1	1

#### *Development of Antibodies in Cases of Meningitis in Adults*

As may be seen in Table 2, antibody titres here are not increased to the same degree as in mothers of children infected with *Listeria*. In 4 cases out of 6, elevated titres are nevertheless present higher than 1/32 in the agglutination test and 1/30 or higher in the complement

likely that in these patients the early antibacterial therapy was responsible for the low titre values. In cases 15 and 18, the complement-fixing antibody does assume the form of a curve but the values attained do not amount to the afore-mentioned limit values.

The methods employed have thus proved useful in illustrating the development of antibodies in bacteriologically verified cases of listeriosis.

### DISCUSSION

Purulent meningitis in both adults and newborn children is a serious disease which, in the absence of antibacterial therapy, may lead to death. The fact that apparently healthy mothers may give birth to a child or foetus with listeriosis, infected in utero or at parturition, seems to indicate that the mothers have actually been infected themselves although with negligible symptoms. There are thus certain similarities between listeriosis and toxoplasmosis in pregnancy. In toxoplasmosis serological methods have been of considerable help in the diagnostic procedure and it is therefore to be hoped that such methods in the future will play an equally important rôle in establishing elevated antibody titres against *L. m.*

The methods applied in this investigation to determine O agglutinins and complement-fixing antibodies against *L. m.* proved elevated antibody titres to develop in most cases provided that no early antibacterial therapy is given that reduces the stimulating effects of antigen on antibody formation. It was also found that on the whole these antibody curves agree with those of other specific antibodies in bacterial diseases, except that the elevated titres tend to remain for a long time.

By the same procedure, no certain cross-agglutination between *L. m.* O antigen on the one hand and *Staphylococcus aureus* or enterococci on the other could be demonstrated but in spite of this, it is possible that complement fixing antibodies may be more specific than agglutinins, as was proposed by Seeliger. Further methods for a serological diagnosis of human listeriosis are to be awaited and hoped for.

### SUMMARY

The development and successive course of O agglutinins and complement-fixing antibodies have been studied in 14 mothers of full term or abortive children with listeriosis, and in 6 adult cases of Listeria meningitis. In 11 of the mothers the O agglutination titre rose to 1/64 or more, and in 11 cases out of 9 the complement fixation titre was 1/30 or more. The antibody curves in the mothers developed in a manner similar to that of specific antibodies in other bacterial diseases but were characterized by a slow retardation. In these cases antibacterial therapy had not been administered during the acute stage. Six cases of purulent meningitis in adults with *L. m.* were studied, and in four of these an

fixation test Only in two of these six cases is there a simultaneous increase of both antibodies These patients had all received intensive chemotherapeutic and antibacterial treatment in the acute stages of their illness It may reasonably be presumed that such therapy resulted in a less effective antigenic stimulation of the organs responsible for antibody formation so that the rate of immunization is markedly reduced in comparison with that in mothers of children with listeriosis

### *The Antibody Curves*

Fig 1 illustrates the development of the antibody titres in 11 mothers of Listeria-infected foetus or child As might have been supposed a well-defined antibody curve is obtained In its later stages the antibody curve falls off very slowly proving the elevated titres to persist for several months In the acute phase on the other hand, the titre rises rapidly as evidenced by cases 6, 8, and 11 which also serve to exemplify the typical features of the later part of the antibody curve In cases 11 and 13 we may see the tendency for the complement fixation titre to increase later than the agglutination titre But in all important respects these antibody curves possess the same characteristics as those found in other bacterial diseases, although the comparatively slower retardation should be noted Since most of these mothers did not receive antibacterial therapy it may be suspected that the antigen was allowed to act for a considerable period of time thus sustaining the elevated antibody titre

Only three of the adult meningitis cases are useful for illustrative purposes and are shown in Fig 2 Here the antibody curves are lower but the trend is similar to that of other bacterial diseases It seems

#### Antibodies in adult cases of human listeriosis

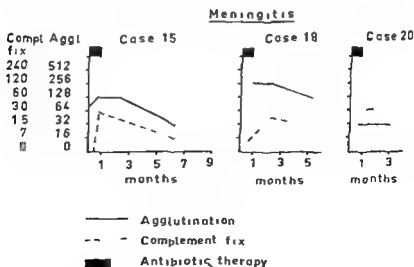


Fig 2

## STUDIES OF ANTIBODIES IN HUMAN LISTERIOSIS

### 2 *Antibody Titres in Healthy People and Patients of Different Categories*

By

STEN WINBLAD and N. E. BORGLIN

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If animals are immunized with *Listeria monocytogenes* (L.m.) they respond with elevated titres of O agglutinins and complement-fixing antibodies. Also human cases of listeriosis show such an increase of the two types of antibody and this response is especially marked in women with newborns or abortions infected with L.m. (Winblad). It should be added that the antibody titre curves are best developed in the absence of antibiotic therapy.

The possible existence of a serological cross reaction between agglutinins against L.m. and *Staphylococcus aureus* or enterococci (Seeliger) has caused some debate whether a high agglutination titre should necessarily be taken as specific proof of an infection with L.m. In the opinion of Seeliger, complement-fixing antibodies provide more valuable information.

In the present investigation where O-antigens of the five different types (1, 2, 3, 4a, 4b) were used for agglutination reactions, and whole antigens of these strains were employed in complement fixation tests, no evidence of any cross reaction has been noted with antigens of *Staphylococcus aureus* or enterococci (Winblad). Our findings are thus in agreement with those of Welshimer.

It is important to find out if subclinical infections with L.m. are frequent and if they give rise to increased agglutination titres in comparatively healthy people. If this should be the case, the responsible antibody is nevertheless to be regarded as "specific". In Germany, Seeliger found agglutination titres of 180 or more in 33 per cent of the human sera he examined. In the same country, Pöschel & Schwind studied 504 healthy pregnant women and found agglutination titres of 160 in 24.2 per cent. In consequence, these investigators consider high agglutination titres to be of diagnostic value only when associated with definite clinical symptoms.

In USA, Osbold & Sawyer found an agglutination titre of 1/50 (for-

elevated O agglutination was found as well as an increased complement fixation titre

No cross agglutination against O antigen from *Staphylococcus aureus* or enterococci could be demonstrated with the methods employed. However, the complement-fixing antibody may be the more specific one

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animals it seemed desirable to find out whether poultry farm workers have increased antibody titres. Consequently, serum samples were collected from 30 such persons. The result is presented in Table 1, showing that none of the persons of this study attain a positive or even weakly positive value. Although the investigated material was not very large there seems to be no reason for suspecting contact with poultry to be a common cause of infection.

TABLE 1  
*Distribution of Antibody Titres in Healthy Persons (in Per Cent)*

Category	Total	O-Agglutination ≥ 64 > 64		Complement fixation ≥ 30 > 30		O-Agglut = 64 Cpl fix or > 30 O-Agglut > 64 Cpl fix = 30 (+)	O-Agglut > 64 Cpl fix > 30 +	Column 5+6
Blood donors	240	13	17	22	95	21	0.8	29
Poultry farm workers	30	67	0	67	0	0	0	0

*Distribution of Antibody Titres in Patients with Mononucleosis infectiosa and Non specified Diseases*

With regard to Nyfeldt's early observation that an epidemic in Copenhagen of a disease closely resembling infectious mononucleosis might have been caused by L.m., 48 sera delivered to the laboratory from patients with mononucleosis infectiosa and with a positive Paul-Bunnell's reaction were examined for antibodies against L.m. However, in these cases no positive or weakly positive titres were found (Table 2). At the same time, examinations were made of 98 cases of pharyngitis showing clinical resemblance to mononucleosis infectiosa, but giving a negative Paul-Bunnell's reaction. In none of these cases, either, was a positive titre to be found. It is thus apparent that an infection with L.m. should not be regarded as commonly associated with mononucleosis infectiosa.

Also recorded in Table 2 is an examination of sera from 100 different patients, treated at the department of internal medicine in Malmö. These patients were suffering from various diseases but only 2 cases showed titres designed positive according to the criteria stated above. A similar investigation was performed in a hospital situated in a rural area (Hörby) where the population consists largely of farmers who to a large extent have very close contacts indeed with poultry. 76 patients of this latter group were examined, and only in one case (1.3 per cent) was there an elevated titre, and this titre was encountered in a sailor who had just returned from tropical waters. It is thus evident that not even this last group lends any support to the theory that a serologically verifiable contact with L.m. should be associated with persons in frequent contact with poultry.

malinized antigen) in 53 per cent of normal subjects and consider a moderate titre of *e.g.* 1/25 to be so common as to be regarded 'normal'

As may be seen from our previous report, in cases of bacteriologically verified human listeriosis there are increased titres of O-agglutinins and complement-fixing antibodies especially in the absence of anti-bacterial therapy. Antibody curves are produced that are in full agreement with those of other bacterial infections. A new investigation therefore seemed called for in which the same technique was employed but this time on a large material including both healthy subjects and patients suffering from various diseases. It was hoped that such a study would throw light on the average amount of such antibodies in the Swedish population and also on the frequency with which the disease tends to appear in Sweden.

### METHODS

The preparation of antigens and the techniques of the O agglutination and complement fixation reactions have been described in the first part of this study (Wahlblad).

Sera from healthy and diseased subjects tabulated below in connection with the results obtained were examined after inactivation at 56° C for 30 minutes.

### RESULTS

#### *Distribution of Antibody Titre in Healthy Persons*

Blood donors are taken here to represent healthy individuals, since, in order to be accepted as donors, they are subjected to ESR and Wassermann tests, and the state of their blood and lungs is controlled. Table 1 shows that among 240 such blood donors, 87 per cent had an agglutination titre of less than 1/64, 11.3 per cent had a titre of 1/64 and 1.7 per cent had a higher titre. As for complement-fixation, 77.2 per cent had a titre of less than 1/30, 13.3 per cent a titre of 1/30 and 9.5 per cent had a higher titre. In consideration of this distribution it seems justified to regard O-agglutination titres higher than 1/64, and complement-fixation titres higher than 1/30 as sufficiently increased, compared to normal conditions, to be taken as indicative of or of a recent or actually present contact with Lm. A combination of elevated titres in both reactions is marked out with a positive sign in the table, whereas an increased titre of either antibody combined with the limit value of the other is considered weakly positive. On this assumption, 2.9 per cent of the blood donors are found to have an increased antibody titre against Lm.

The frequency of *Listeria monocytogenes* in animals sent in for autopsy to the State Veterinary Institute of Sweden has been reported by Nilsson & Karlsson. In the years 1948-1957 they found these bacteria in 141 instances, and of these no less than 111 were poultry. Although this high contribution on the part of the poultry may possibly be due to the fact that they are more frequently sent in for autopsy than other

first of the groups tabulated here comprises cases where the pregnancy took a normal course and the baby showed no signs of illness when leaving the maternity clinic. The investigation was started after the occurrence of a few cases of listeriosis and includes 2 271 successive maternity cases, no selection attempted. Throughout this whole investigation, no single case of bacteriologically verified listeriosis was found in the clinic. It will be seen from the table that the percentage of cases showing an elevated antibody titre is small (1.1 per cent) being closely similar to the previously studied normal material. In spite of this low percentage, though, it should be noted that there are mothers who have an elevated titre but who nevertheless showed no signs of illness and gave birth to apparently healthy children. As was the case with the previously described normal material it must be supposed that such an elevated titre may be the result of a sub-clinical infection at a date that cannot be determined. No antibiotic therapy was given on the strength of these findings alone, and the pregnancy was allowed to take its normal course. There is thus no reason to suspect that a newborn child is going to have listeriosis merely because its mother shows an elevated antibody titre. Another interesting thing that emerged from this investigation was the fact that several of these women who had an elevated antibody titre were immigrants from Central Europe. Possibly the infection is more common in other European countries than in Sweden where, after all, the risk seems comparatively small.

86 cases of imminent abortion were examined, too, but none of these showed antibody titres higher than the supposed limit values.

The third group consists of 102 non-specified abortions—that is, there was no special selection, every woman who happened to have an abortion during the period of investigation was subjected to a serological examination. Within this group, there is a weak but no significant tendency for the women to have higher antibody titres than the large group of normal pregnant women.

The next three groups recorded in Table 3 are "selected", however. These sera do not originate from the Malmö clinic but were obtained from other Swedish maternity clinics where some obstetrical complication had occurred and an infection, e.g. listeriosis, seemed a conceivable explanation. As distinct from the first three groups of this material where entirely nonselective principles were abided to, these three groups may be said to have been specially chosen on the suspicion of a possible infection. Within this last group of abortions, a considerable number of the women

showed elevated antibody

titres. Of the

100 per cent have an

antibody titre, and the mothers of diseased or deformed children have increased titres in 12.9 per cent. There is obviously a much higher percentage of elevated antibody titres in these groups than in the normal material. It is probable that in many of these cases listeriosis



TABLE 2

*Distribution of Antibody Titres in Patients with Mononucleosis infectiosa and Non specified Diseases (in Per Cent)*

Patient category	Total	O Agglutination		Complement fixation		O Agglut = 64 Cpl fix = 30 or H Agglut > 64 (pl fix = 30 (+))	O Agglut > 64 Cpl fix +	Count n = 6
		> 64	> 64	> 30	> 30			
Mononucleosis infectiosa (Positive Paul Bunnell reaction)	48	27.8	6.3	14.6	6.3	0	0	0
Pharyngitis (Negative Paul Bunnell reaction)	88	7.1	1.0	11.2	6.1	0	0	0
Non specified disease (City of Malmö)	100	8	0	6	4	2	0	2
Non specified diseases (Rural district) (Hörby)	76	13.8	2.6	11.8	9.2	1.3	0	1.3

TABLE 3

*Distribution of Antibody Titres in Obstetrical Patients in Various Situations (in Per Cent)*

Patient category	Total	O Agglutination		Complement fixation		H Agglut = 64 Cpl fix = 30 or O Agglut > 64 Cpl fix +	O Agglut > 64 Cpl fix +	Count n = 6
		> 64	> 64	> 30	> 30			
Normal pregnancies with healthy offspring	2271	17.2	4.7	8.5	3.5	0.2	0.9	11
Imminent abortions	86	15.1	2.3	2.3	0	0	0	0
Abortions (non specified Malmö)	102	19.1	7.8	8.8	9.9	1.9	1.9	3.8
Abortions (unknown causes suspected infections)	121	43.5	20.5	29.7	12.4	10.7	5.8	16.5
Mothers of children dead in the perinatal period	158	30.7	12.4	17.8	7.7	4.8	3.6	8.4
Mothers of diseased or deformed children	85	34.4	19.5	22.3	8.2	8.2	4.7	12.9

### *Antibody Titres of Obstetrical Patients in Various Situations*

Since listeriosis plays an important part especially among the diseases of newborn children it seemed desirable to establish the frequency of elevated antibody titres in normal pregnancies as well as various obstetrical complications, such as abortion imminent abortion death of the child in the perinatal stage or illness during the neonatal period. The result of such an investigation is set forth in Table 3. The

is combined with a complement fixation titre above 1/30 or a complement fixation titre of at least 1/30 with an O agglutinin titre above 1/64.

There is no evidence from this investigation that persons occupied with poultry farming or otherwise belong to the rural population have higher antibody titres than other people. The road which L. m. may take from animal to human hosts is still unknown.

Vyfeldt's proposition that mononucleosis infectiosa might have some relation to an infection with L. m. was in no way substantiated by our results. Nor is there any reason to believe that cases of pharyngitis of similar descriptions but with a negative Paul Bunnell's reaction have any connection with L. m.

In cases of uncomplicated pregnancies in our country there seems to be no greater frequency of increased antibodies against L. m. than in other persons (2-4 per cent).

On the other hand elevated antibody titres are comparatively frequent (8-16 per cent) in women whose pregnancies have been complicated by abortions of unknown aetiology or perinatal death of the children or whose newborns have been diseased or deformed from presumably infectious causes. Here an infection with L. m. is often a possible explanation. It should be kept in mind, however, that 2-4 per cent may have elevated titres but show no clinical signs of listeriosis. Such an elevated titre is therefore no proof of actual clinical listeriosis but should nevertheless be noted. Pregnant women with increased titres and the newborn children of such mothers should also be kept under close observation since any signs of meningitis on the part of the child urge immediate antibiotic therapy.

The previous reported material containing sera from diagnosed cases of human listeriosis also shows that antibodies will develop if no early antibiotic treatment is given. Furthermore it proves serological findings to be good evidence of recent or actually present infections with L. m.

#### SUMMARY

Healthy subjects and groups of patients suffering from different diseases were examined for elevated titres of antibodies against L. m. O agglutinin titres above 1/64 and complement fixing antibodies in titres above 1/30 are regarded as significantly increased and suggestive of an infection with L. m. Such an elevated value in combination with the limit value of 1/64 in the case of O agglutination or 1/30 in the case of complement fixation is interpreted in a similar manner.

In 240 healthy blood donors elevated titres occurred in 2.9 per cent. In 48 cases of mononucleosis infectiosa there was no single case of an elevated antibody titre against L. m. Nor could any elevated titres be demonstrated in 98 patients with pharyngitis but with a negative Paul Bunnell's reaction. In patients suffering from various internal dis-

lies behind the abortion or the perinatal complication. It is not intended to analyze individual cases here, that will be done in a later report.

It will be realized from what has just been said that listeriosis seems a reasonable explanation of several cases of obstetrical complications with abortions and perinatal deaths. As a consequence, it is important for diagnostic purposes to make cultivations on material from the vagina, abortions, and dead infants, and to study the course and type of the antibody curves in the mothers.

### *Antibody Titres in Cases of Human Listeriosis*

Previously reported, diagnosed cases of listeriosis (Winblad) are mentioned here for comparison with the material described above. In mothers of newborns or abortions, the frequency of elevated antibody titres calculated according to the criteria used in this study, amounts to 88 per cent. Although the number of cases examined is not very large it warrants the conclusion that elevated antibody titres accompany the infection. In these mothers no antibiotics were administered at the early stage of the obstetrical complication, and for this reason the development of antibodies remained unaffected. In the case of meningitis caused by *L. m.*, on the other hand, antibiotic treatment was started very soon and this probably explains why only 1/3 of these cases have an antibody titre higher than the calculated limit values.

### DISCUSSION

Clinical infections with *Listeria monocytogenes* occur in Sweden, although they are relatively rare. However, titres of O-agglutinins below 1/64, and complement-fixing antibodies below 1/30 are by no means uncommon in healthy people in this country. Only in 2-4 per cent, though, are these limit values exceeded, that is an O-agglutination titre of 1/64 in combination with a complement-fixation titre of more than 1/30, or a complement-fixation titre of 1/30 in combination with an agglutination titre of more than 1/64. If we regard these combinations as typical of a recent or actually present infection with *L. m.*, such elevated titres can only be demonstrated in those 2-4 per cent. It is an open question whether titres lower than those mentioned are indicative of a cross immunity against other antigens among Gram-positive bacteria or whether they are evidence of a subclinical infection with *L. m.* Provided that the titres are not a manifestation of any cross antigenicity the conclusion must be that latent and subclinical infections with *L. m.* are comparatively common but do not usually lead to any considerable increase of antibodies. This finding, however, applies only to Sweden, and in all likelihood similarly performed investigations in South or Central Europe might show elevated antibody titres to be much more frequent. As things stand however, the result is possibly indicative of a clinical infection with *L. m.* if an O-agglutinin titre of at least 1/64

## TRIALS WITH A RAPID METHOD FOR QUANTITATIVE AND QUALITATIVE VIRUS DETERMINATION BY MICROSCOPIC EXAMINATION OF THE INDIVIDUAL CELLS IN VIRUS INOCULATED MONOLAYERS ON SLIDES

By

SVEN BERGMAN, INGRID STENRAM and UÑNE STENRAM

Received 13 XI 62

Microscopic examination of the effect of viruses on cells is generally performed on monolayers cultivated on coverslips or on squashed cells. Recently, a simple method was devised for cultivating monolayers on slides in Hellenenthal cuvettes (*Bergman 1959*). Slides are easy to handle and the possibilities of examining the individual cells are good. The procedure therefore appears to enable a rapid and simple estimation of a virus sample inoculated on a monolayer. A preliminary report of a trial with such a method is given below.

### MATERIAL AND METHODS

#### Media and TCM

Cells: Human amniotic cells prepared according to *Lahelle (1956)*. All cells used in one experiment were from the same placenta.

Viruses: Adenovirus type 7 (Ad-7) and Coxsackievirus B<sub>1</sub> were supplied by the State Serum Ins. The viruses were supplied by the viruses are given in the Tables 4-5.

Experimental procedure: The cells were fixed on a slide by of cell suspension.

The cells were then washed with distilled water. The viruses were added to the slides simultaneously with the cells. The cells and viruses were added to the slides simultaneously.

Attachment of the cells and adsorption of the viruses took place with the slides horizontal in moist air with 8 per cent CO<sub>2</sub> in a covered chamber at 37° C. After

The investigation was supported by grants from the Swedish Medical Research Foundation and the Medical Faculty of Lund.



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## MATERIAL AND METHODS

**Media** TCM 199 with 20 per cent pooled human inactivated serum or 20 per cent rabbit serum or 100 µg/ml penicillin and 100 µg/ml streptomycin for maintenance medium against adenovirus 7 (expressed as serum of 100 TCD<sub>50</sub> of virus).

**Cells** Human amniotic cells prepared according to Lahelle (1956). All cells used in one experiment were from the same placenta.

**Viruses** Adenovirus 7, given by the Statens Serum Institut, Copenhagen.

**Experiment** The cells were grown on slides and fixed on a slide.

Attachment of the cells and adsorption of the viruses took place with the slides horizontal in moist air with 8 per cent CO<sub>2</sub> in a covered chamber at 37°C. After

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seases, an elevated antibody titre against *L. m.* was found in 2 per cent and in a rural population, similarly examined, in 1.3 per cent. Persons dealing with poultry do not have antibody titres against *L. m.* higher than normal.

In normal pregnancies as well as in a series of undifferentiated abortions, an elevated antibody titre was demonstrated in 1.1 per cent only. In a selected material of abortions from unknown, but probably infectious causes, or of mothers of infants that died in the perinatal period or of mothers with diseased or deformed children, however, the amount of serologically positive cases was considerably increased (8.16 per cent), and in these last groups it must be supposed that some of the obstetrical complications were caused by *L. m.*

Healthy pregnant women who gave birth to living, healthy babies showed the same percentage of increased antibody titres as other healthy subjects. An infection with *L. m.* although stimulating a development of antibodies may thus not always produce any clinical disorder in the child. Also, the infection indicated by the elevated antibody titre may belong to the past.

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various intervals (given in the Tables) the fluid was pipetted off and the rings were removed. The slides were carefully washed in medium as a rule the one containing 20 per cent pooled human inactivated serum and placed vertically into Hellenthal cuvettes containing this medium. Each cuvette accommodated 3 slides. The loaded cuvettes were placed in moist air with 8 per cent  $\text{CO}_2$  at  $37^\circ \text{C}$ .

Check examination of the supernatant and the monolayers revealed that 80-90 per cent of the deposited cells had attached to the slides.

*Cytological methods* Slides were removed from the cuvettes at different intervals (see the Tables), washed in physiological saline ( $37^\circ \text{C}$ ) for 3 minutes and fixed in

1. non specific changes (cf. Dunnebocke 1956). In the early stages of specific cell degeneration caused by poliomyelitis virus type 3 (Fig. 3) the nuclei were wrinkled and the nucleoli could often not be identified. In these stages the cytoplasm was still of normal outline though it was often vacuolized and sometimes contained basophil granules. Sometimes the cytoplasm showed small spherical projections. In later stages in which the changes could not be distinguished from non specific degeneration the cells were rounded and showed no such projections while the

under a  $\times 100$  examined. Those cells with specific changes were then seen were marked 0+ in the tables. When the cells had become attached to the slides

Examination of a slide required 5-10 minutes

## EXPERIMENTS OBSERVATIONS AND DISCUSSION

### Adsorption of Viruses

The adsorption experiments were carried out with amniotic cells which were allowed to attach to the slides for 1 hour. The preparation was then inoculated with viruses which were given  $\frac{1}{2}$ , 2 or 6 hours to adsorb to the cells. The results are given in Tables 1 and 2.

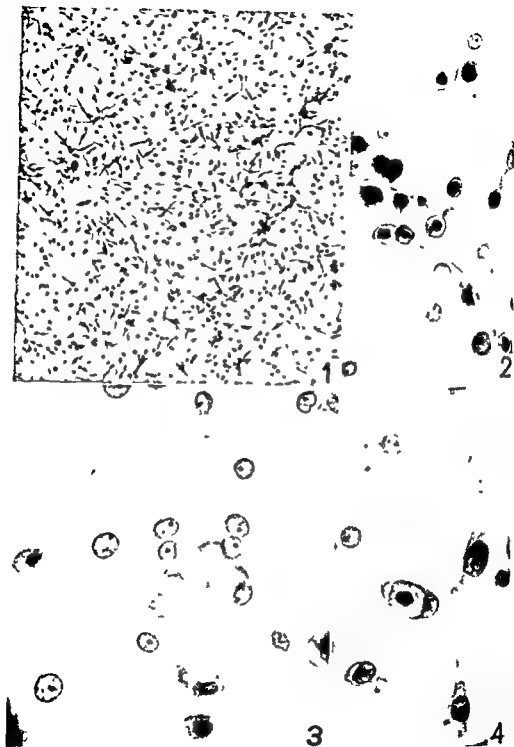
Fig. 1 Control culture 4th day  $\times 100$

Fig. 2 Culture inoculated with adenovirus type 7 4th day 10 cells with specific cytopathic changes are seen in focus  $\times 420$

Fig. 3 Culture inoculated with poliomyelitis virus type 3 2nd day 7 cells with specific cytopathic changes are seen in focus  $\times 420$

Fig. 4 Control culture 2nd day Centrally a cell with non specific degeneration  $\times 420$





*Figs 1-4*

Cultures of human amniotic cells fixed in 96 per cent alcohol  
and stained with Htx eosin

amniotic cells within 6 hours but not within 2 hours. This assumption is in good agreement with the findings of Ginsberg (1958a), Lieberman & Friedman (1959), and Pereira (1959). The absence of any effect of the supernatant fluid after an adsorption period of 6 hours might also be explained by the assumption that viruses not adsorbed within this period are inactivated. However, tests in which the primary virus material was allowed to be adsorbed for 6 hours to slides without cells still showed full activity in the supernatant.

TABLE 3

*Amount of Virus Adsorbed after Various Time Intervals*

Aden. virus Type 7 Titre log TCID<sub>50</sub> 0.1 ml 40 100 000 Cells per Slide

	2 h	supern	total	6 h	supern	total
A	376	432	808	680	0	680
	458*	531	989	736	1	737
B	343	373	716	713	0	713

Read on sixth day

In "A" the cells were allowed 1 hour to become attached to the slides before

\* =

TABLE 4

*Amount of Virus Adsorbed after Various Time Intervals*

Measles Virus Type 3 Titre log TCID<sub>50</sub> 0.1 ml 34 100 000 Cells per Slide  
See also Table 3

	2 h	supern	total	6 h	supern	total
A	42	114	156	90	23	113
	44	113	157	112	21	133
B	13	95	108	117	15	132

Read on sixth day

On comparison of the data given in Tables 1 and 3 it will be seen that a difference was found between the effect of the virus after 2 hours and 6 hours respectively in the experiments accounted for in Table 3 but not in the experiments summarized in Table 1. The virus dose used was the same in both experiments. In the one experiment (Table 1) the number of cells was 60 000 per slide as against 100 000 in the other (Table 3). The amniotic cells used in one experiment originated from one placenta and those used in the other from another placenta. This may help to explain the difference mentioned as it is known that the susceptibility of amniotic cells to viruses may vary from one placenta to another (Osterhaut & Tamm 1959). It should also be remembered

TABLE 1  
*Adsorption Time of Viruses*

Adenovirus Type 7, Undiluted Titre log TCD 50/0.1 ml-4.0 60 000 Cells per Slide  
Table Gives Number of Cells per 1,000, with Specific Cytopathic Changes

Adsorption time in hours	Interval in hours after commencement of inoculation			
	20	45	68	94
½	1	183	279	311
2	5	405	695	607
6	4	490	503	689

TABLE 2  
*Adsorption Time of Viruses*

Poliomyelitis Virus Type 3 Undiluted Titre log TCD 50/0.1 ml-4.6 60 000 Cells per Slide  
Table Gives Number of Cells per 1 000, with Specific Cytopathic Changes

Adsorption time in hours	Interval in hours after commencement of inoculation			
	20	45	68	94
½	24	83	130	52
2	44	252	222	262
6	275	680	677	741

As regards adenovirus type 7 (Table 1), the number of cells with specific cytopathic changes increased slightly on prolongation of the adsorption period from 2 to 6 hours. When the virus had been allowed only ½ hour for adsorption, the effect was considerably lower. The effect of poliomyelitis virus type 3 increased with the adsorption time (Table 2).

To test how complete virus adsorption was after 2 and 6 hours, supernatant fluid was pipetted off at these intervals. The fluid was added to suspensions of cells from the same population as that used in the primary test. The cells were allowed to attach on slides. After 0 hours the supernatant was pipetted off, and the slides were washed in medium with 20 per cent pooled human inactivated serum and placed in Hellenthal cuvettes containing this medium. The cuvettes were handled as described above. At different intervals the slides were taken for cytological examination. The results are given in Tables 3 and 4.

The results in Table 3 show that primary adsorption of adenovirus type 7 for 6 hours but not for 2 hours gave a supernatant fluid, which, as judged by the same method on new slides was completely or almost completely free of infectious viruses. The sum of the effect caused by the primary virus sample after an adsorption time of 2 hours and its supernatant fluid was nearly the same as the virus effect of 6 hours' primary adsorption. The findings suggest that under the conditions used adenovirus type 7 is completely or almost completely adsorbed to

amniotic cells within 6 hours but not within 2 hours. This assumption is in good agreement with the findings of Ginsberg (1958 a), Lieberman & Friedman (1959), and Pereira (1959). The absence of any effect of the supernatant fluid after an adsorption period of 6 hours might also be explained by the assumption that viruses not adsorbed within this period are inactivated. However, tests in which the primary virus material was allowed to be "adsorbed" for 6 hours to slides without cells, still showed full activity in the supernatant.

TABLE 3

Amount of Viruses Adsorbed after Various Time Intervals  
Adenovirus Type 7 Titre log TC<sub>50</sub> 0.1 ml ~4.0 100,000 Cells per Slide

	2 h	supern	total	6 h	supern	total
A	376	432	808	680	0	680
	458*	531	989	736	1	737
B	343	373	716	748	0	748

Read on sixth day

In "A" the cells were allowed 1 hour to become attached to the slides before  
a. 1st on 5th

TABLE 4

Amount of Viruses Adsorbed after Various Time Intervals  
Poliovirus Type 3 Titre log TC<sub>50</sub> 0.1 ml 3.6 100,000 Cells per Slide  
See also Table 3

	2 h	supern	total	6 h	supern	total
A	42	114	156	90	23	113
	44	113	157	112*	21	133
B	14	88	102	117	13	130

Read on sixth day

On comparison of the data given in Tables 1 and 3 it will be seen that a difference was found between the effect of the virus after 2 hours and 6 hours, respectively, in the experiments accounted for in Table 3 but not in the experiments summarized in Table 1. The virus dose used was the same in both experiments. In the one experiment (Table 1) the number of cells was 60,000 per slide as against 100,000 in the other (Table 3). The amniotic cells used in one experiment originated from one placenta and those used in the other, from another placenta. This may help to explain the difference mentioned, as it is known that the susceptibility of amniotic cells to viruses may vary from one placenta to another (Osterhout & Tamm 1959). It should also be remembered

TABLE 1  
*Adsorption Time of Viruses*

Adenovirus Type 7, Undiluted Titre log TCD 50/0.1 ml -4.0 60 000 Cells per Slide  
Table Gives Number of Cells, per 1,000 with Specific Cytopathic Changes

Adsorption time in hours	Interval in hours after commencement of inoculation			
	20	45	68	94
1/2	1	183	279	311
2	5	405	695	607
6	4	490	503	689

TABLE 2  
*Adsorption Time of Viruses*

Poliomyelitis Virus Type J Undiluted Titre log TCD 50/0.1 ml -4.6 60 000 Cells per Slide  
Table Gives Number of Cells per 1,000, with Specific Cytopathic Changes

Adsorption time in hours	Interval in hours after commencement of inoculation			
	20	45	68	93
1/2	24	83	130	52
2	44	252	222	262
6	275	680	677	741

As regards adenovirus type 7 (Table 1), the number of cells with specific cytopathic changes increased slightly on prolongation of the adsorption period from 2 to 6 hours. When the virus had been allowed only 1/2 hour for adsorption, the effect was considerably lower. The effect of poliomyelitis virus type 3 increased with the adsorption time (Table 2).

To test how complete virus adsorption was after 2 and 6 hours, supernatant fluid was pipetted off at these intervals. The fluid was added to suspensions of cells from the same population as that used in the primary test. The cells were allowed to attach on slides. After 6 hours the supernatant was pipetted off, and the slides were washed in medium with 20 per cent pooled human inactivated serum and placed in Hellenthal cuvettes containing this medium. The cuvettes were handled as described above. At different intervals the slides were taken for cytological examination. The results are given in Tables 3 and 4.

The results in Table 3 show that primary adsorption of adenovirus type 7 for 6 hours but not for 2 hours gave a supernatant fluid, which as judged by the same method on new slides was completely or almost completely free of infectious viruses. The sum of the effect caused by the primary virus sample after an adsorption time of 2 hours and its supernatant fluid was nearly the same as the virus effect of 6 hours' primary adsorption. The findings suggest that under the conditions used adenovirus type 7 is completely or almost completely adsorbed to

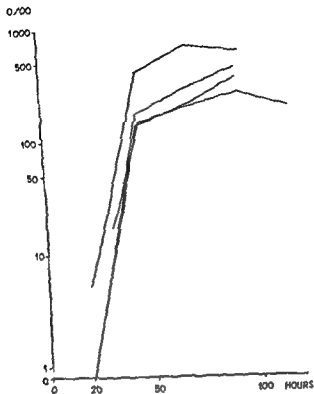


Fig 5

1000 with specific  
it Virus titre log  
are accounted for

(cf under Material and Methods) Non-specific cell degeneration was seen in the controls generally in about the same percentage as in high virus dilutions

#### *Titration of Virus*

Experiments were performed with poliomyelitis virus type 3 and adenovirus type 7 in various dilutions to study a possible quantitative relationship between inoculated virus and the number of cells with specific cytopathic changes (see Tables 5 and 6)

The tube technique is more sensitive than the slide technique at high virus dilutions. That is certainly due to the fact that attack on a single cell in the tube results in the breakdown of the whole culture according to the all or-none principle. If secondary spread of viruses is restrained, as seems to be the case with our slide technique (see below), the attack

that not all cells in a population are susceptible to viruses (Dulbecco 1958, and others). When, therefore, as in the experiments accounted for in Table 1, the virus produces a strong effect already after 2 hours adsorption, the effect can hardly be expected to increase much on increase of the dose or extension of the adsorption period.

Poliomyelitis virus type 3 had stronger effect after an adsorption period of 6 hours than of 2 hours, but after both periods the supernatant still contained infectious viruses (Table 4). This was presumably due to incomplete adsorption of viruses. Previous investigators have also found that if poliomyelitis virus is allowed 1 hour in which to be adsorbed to monkey kidney tissue cells and the cells are then thoroughly washed, poliomyelitis virus will still be demonstrable in the medium during the first few hours (Reale *et al.* 1956, Reissig *et al.* 1956). A release of new-formed poliomyelitis virus and an increase in the virus titre of the medium has been demonstrated for HeLa cells 6 hours after inoculation (Lwoff *et al.* 1955), for monkey kidney cells 5-11 hours after inoculation (Reale *et al.* 1956, Reissig *et al.* 1956) but not until 8 hours after inoculation for amniotic cells (Dunnebacke 1956). Since amniotic cells were used in the present investigation and since the poliomyelitis virus activity in the supernatant was lower after an adsorption time of 6 hours than of 2 hours, it is probable that no more than a negligible amount of new-formed viruses were released during the period of adsorption.

#### *Interval between Inoculation and Reading of Result*

The cultures were examined at various intervals after inoculation but not later than on the seventh day. Adenovirus type 7 was found to produce cytopathic changes in only a few cells within the first 24 hours. On the second day, however, the changes in the cell cultures were marked. There was a slight increase in the number of affected cells on the third day. The extent of the alterations persisted unchanged on the fourth day (Tables 1 and 5 and Fig. 5).

The tests with poliomyelitis virus are given in Tables 2 and 3 and Fig. 6. As mentioned under Material and Methods, the changes caused by the virus could not always in late stages be distinguished with certainty from non-specific cell degeneration. The numbers of cells showing signs of non-specific degeneration are therefore included in brackets in Table 6. It is clear from the table that the number of cells with specific cytopathic changes was fairly constant from the second to the fifth day inclusive.

It is also seen in Table 6 that the number of cells with changes of unspecific appearance was largest in the slides inoculated with virus in high concentrations. The explanation is probably that some of the cells showing apparently unspecific changes were in fact degenerating owing to virus infection, though this could not be recognized in late stages.

TABLE 5  
*Effect of Virus Dilution*

Adenovirus Type 7 Titre log TCD 50 0.1 ml -4.5 100 000 Cells per Slide Adsorption Time 2 Hours Table Gives Number of Cells per 1 000, with Specific Cytopathic Changes

Experiment	Virus dilution	Interval in days after commencement of inoculation					
		1	2	3	4	5	6
A	10 <sup>0</sup>	0	139	214	360		
	10 <sup>-1</sup>	0	49	51	117		
B	10 <sup>0</sup>	0	175	301	442		
	10 <sup>-1</sup>	0	60	57	104		
C	10 <sup>0</sup>	16	143	207	262	193	
	10 <sup>-1</sup>	1	1	12	13	23	13
	10 <sup>-2</sup>	0	0	1	0+	3	0+
	10 <sup>-3</sup>	0	0	0	0	2	0+
	10 <sup>-4</sup>	0	0	0	0	0	0

TABLE 6  
*Effect of Virus Dilution*

Poliomyelitis Virus Type 3 Titre log TCD 50 0.1 ml -3.5 100 000 Cells per Slide Adsorption Time 6 Hours Table Gives Number of Cells per 1,000, with Specific Cytopathic Changes Bracketed Figures Indicate Number of Cells, per 1 000, with Non Specific Changes

Virus dilution	Interval in days after commencement of inoculation						
	1	2	3	4	5	6	7
10 <sup>0</sup>	32(29)	47(38)	50(16)	64(18)	64(26)	51(93)	43(102)
10 <sup>-1</sup>	0+(17)	1(6)	4(2)	4(3)	8(12)	9(12)	0(75)
10 <sup>-2</sup>	0(17)	0+(7)	0(6)	0+(10)	1(3)	0+(6)	3(21)
10 <sup>-3</sup>	0(5)	0(9)	0(4)	0(2)	0+(4)	0(4)	0(5)
10 <sup>-4</sup>	0(9)	0(1)	0(4)	0(8)	1(9)	0(6)	0(67)
10 <sup>-5</sup>	0(19)	0(5)	0(2)	0(2)	0(6)	0+(16)	0(59)
Controls	0(12)	0(8)	0(7)	0(9)	0(2)	0(11)	0(33)

The differences between experiments A, B and C in Table 5 are probably explained by the different virus susceptibility of amniotic cells from various placentas (Osterhaug & Tamm 1959)

The results with our slide technique (see Figs 5 and 6) indicate that no major spread of viruses occurred during the observation periods. That was certainly due to the medium employed, pooled human serum, which contained antihodies and, may be, other substances inhibiting poliomyelitis virus type 3 and adenovirus type 7. When rabbit normal serum was employed in the maintenance medium there was a stronger effect on the culture, especially in the experiments with poliomyelitis virus type 3, indicating secondary spread of viruses (unpublished experiments). To obtain a true relationship between the number of affected cells and deposited infectious virus particles there should be no



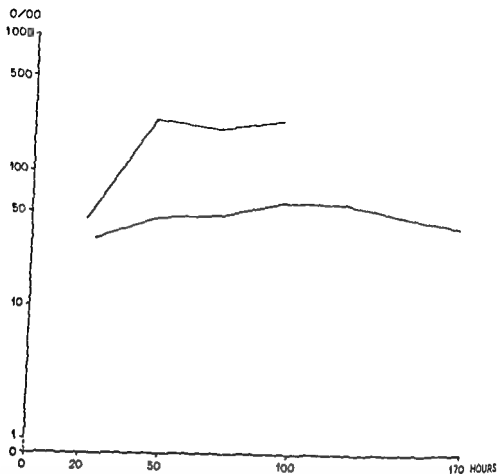


Fig 6

Poliomyelitis virus type 3. The fig. gives in log scale number of cells per 1000 with specific cytopathic changes. Each line represents a separate experiment. Virus titre in the experiment of the upper line log TCD 50 0.1 ml 4.6 and adsorption time 2 hours. Virus titre in the experiment of the lower line log TCD 50 0.1 ml 3.5 and adsorption time 0 hours. The experiments are accounted for Tables 2 and 6.

on a single cell must be difficult to detect. Another factor which may be of importance for the sensitivity is that in the tube technique the monolayer is examined microscopically before inoculation with the virus. Cell cultures in a good condition are selected and this might influence the number of cells attacked. No such selection is possible in the slide technique if the virus and the cells are deposited on the slides simultaneously.

Dilution of poliomyelitis virus (Table 6) as well as high dilution of the adenovirus (Table 5, exp. C) was followed as expected, by about tenfold reduction in the number of cells showing specific cytopathic changes. In the experiments using large doses and producing marked effects (Table 4, exp. A and B), the effect of the dilutions was inferior to the effects expected from the thus reduced number of viruses inoculated. As mentioned, however, not all cells in a cell population are susceptible to viruses.

Examination of a single slide within 1-3 days is often sufficient to estimate the titre of the virus. The slide technique, as here employed, is, however, not suitable for titration of viruses which spread from cell to cell without passing through the medium or which form multinucleated cells.

The slide technique is less sensitive than the tube technique at low virus concentration, probably because the attack on a single cell must be difficult to detect on a slide.

### SUMMARY

A simple method is described for cultivating viruses in monolayers on slides. Adenovirus type 7 and poliomyelitis virus type 3 have been tested. Pooled human serum was employed in the maintenance medium. The effect of the virus is studied by microscopical examination of the individual cells. The effect of variation in the time of virus adsorption and of the interval between inoculation and reading of the results was studied. The results suggest that the method can be used for a rapid ascertainment of a virus attack and, at least for some viruses, in the estimation of their titre.

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secondary spread of viruses in the cultures. It must be examined for every virus studied if this necessitates the addition of specific antibodies to the medium. Viruses which spread from cell to cell without passing through the medium or which form multinucleated cells are probably not suitable for titration according to the slide technique as here employed.

When trying to estimate the titre of a virus sample by the slide technique a few more points must be considered. The slide must be examined at a time when all affected cells show specific cytopathic changes and none of these has been lost. The number of cells deposited on the slides should not change during the incubation period otherwise the percentage of affected cells will not be constant.

Summing up, it may be concluded that an advantage of titration with our slide technique is that it does not require end-point dilution. Determination of the percentage of altered cells on a single slide at a suitable time may be sufficient to estimate the titre of the virus.

#### GENERAL COMMENTS

Judging from our experience with the slide technique described for cultivation and titration of viruses the following points deserve comment.

Attempts were made to provide optimal environment for the cells. Human serum was therefore employed in the maintenance medium. The occurrence of antibodies or other inhibitory substances in human serum (see for example Ginsberg 1958 b) did not prevent the appearance of specific cytopathic changes but it may prevent secondary spread of the virus within the cultures. The washing of the slide after adsorption of the viruses diminishes the non-specific toxic effects, e.g. of a faecal sample.

Several slides can be placed in the same cuvette. This eliminates differences in pH, temperature, composition of the medium etc. between different cultures of one and the same experimental series. It saves cells and space.

As here employed, the slide technique requires, in contrast to the tube technique, staining of the specimens and cytological examination under a high power microscope. Furthermore, many viruses produce readily recognizable changes in the cells. With the slide technique it is therefore in many cases possible to ascertain whether or not the cells have been attacked by viruses and sometimes to form an idea of the group of virus within 1-3 days.

In contrast to the tube and plaque techniques, using the slide technique it is not necessary to wait until the cells have been broken down with secondary spread of the virus to the environments. The difficulties in differentiating between specific and unspecific cell degeneration must be carefully evaluated for every virus concerned.

ment of different pictures of disease for instance kidney tumours or anaemia instead of leukaemia

The intracellular relationship between the virus and the host has been studied with morphological, biochemical and biophysical techniques. The intra cytoplasmatic localization of the active virus as well as the biochemical composition and activity of the virus particle connects it to the nucleotide metabolism of the cytoplasm. Quantitative alterations in the cellular nucleotide metabolism corresponding to the enzymatic pattern of the virus particle can be demonstrated. These alterations might be related to changes in both the energy metabolism and the polynucleotide synthesis of the leukaemic cell.

Analyses of the base composition of the viral ribonucleoprotein reveal possibilities also for an "epigenetic" action of the virus within the cell. This action, however, does not seem to affect species specific properties of the leukaemic cell for instance its type of haemoglobin or its chromosome morphology.

**Ahlstrom C G** Institute of Pathology University of Lund Lund ROLS SARCOMA IN MAMMALS AND DLCS

**Diderholm H & Wesslen T** Institute of Virology University of Uppsala Uppsala INHIBITION OF POLYOMA VIRUS HAEMAGGLUTINATION BY DIFFERENT TISSUES OF MOUSE AND HAMSTER

A study was made on the interaction between polyoma virus and different tissues of newborn and adult mouse and hamster. Homogenates prepared from a number of different organs were found to inhibit polyoma virus haemagglutination. Homogenates of salivary glands of newborn mouse were most effective (haemagglutination inhibition titre of 1/8192). Also homogenates of brain, kidney and adult salivary glands inhibited haemagglutination in high dilutions (1/512 to 1/1024). Low titres or no inhibition at all were found with homogenates of most other organs.

Homogenized cells had a higher capacity than intact cells to inhibit haemagglutination. Most of the inhibitory substance was found in the microsomal fraction. It was found to be sensitive to RDP and periodate but not to trypsin, chymotrypsin, lipase, ether and chloroform.

The question was discussed to what extent the inhibitory substance consists of a receptor substance to polyoma virus and the influence it may have on the tumour formation.

### Meeting December 1

**Holtiger Margareta** National Bacteriological Laboratory Stockholm ANTIBODIES AFTER POLIO VACCINATION

The antibody status before, during and after vaccination with inactivated polio vaccine has systematically been studied in Sweden since 1937. Discrepancies both quantitative and qualitative were observed between the three different types of polio. Constantly high and stable titres were found against type 2. Against type 1 the antibody response was considerably lower and the antibodies showed a higher tendency to decrease. Against type 3 the primary response except in 1937, showed high values equal to those obtained by type 2. These antibodies though showed the same unstable character as those against type 1.

A control in 1961 of the vaccinees 1937 thus revealed considerably decreased anti-

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## PANEL DISCUSSION ON TUMOUR VIRUSES

(S Gard Moderator)

*Alein G* Institute of Tumour Biology Karolinska Institutet Stockholm A SHORT  
SURVEY OF SOME MAIN PROBLEMS IN MODERN EXPERIMENTAL TUMOUR  
VIROLOGY

*Sjogren O* Institute of Tumour Biology Karolinska Institutet Stockholm  
SPECIFIC CELLULAR ANTIGENS IN VIRUS INDUCED TUMOURS

There is now indications for the presence of tumour specific antigens in tumours induced with polyoma virus SV40 virus Moloneyvirus Gross virus Shope virus and Rous virus. The specific cellular antigenicity appears to be common for all tumours induced with the same virus but differs in tumours induced with different viruses. This is in contrast to the finding that tumours induced with chemical carcinogenesis possess an antigenicity specific for each individual tumour. The implications of these findings for tumour therapy or prophylaxis are that a specific immunological prophylaxis is ruled out in nonviral tumours because of the absence of common specific antigens but might be possible with certain limitations with virus induced tumours.

*Okar Blom A* Institute of Virology University of Helsinki Helsinki Finland  
INTERFERENCE PHENOMENA WITH TUMOUR VIRUSES

*Thorell B* Institute of Pathology Karolinska Institutet Stockholm RELATIONSHIP  
OF VIRUS AND CELL IN EXPERIMENTAL LEUKAEMIA

The formation and development of leukaemic cells from the virus infected target cells in the haemopoietic organ has been studied with cytological as well as quantitative cytochemical methods. The leukaemic cell development is described in comparison to the normal blood cell formation and is characterized *inter alia* by accumulation of cytoplasmic ribonucleoprotein.

The ability of the leukaemia virus to affect other types of target cells than the bone marrow elements depends on the host reactions for instance tolerance or antibody formation. Different host reactions in this respect can lead to the devel

human serum totals—to regenerate the inhibitory effect of the serum. Some of the sera were tested against adenovirus types 1, 2, 3, 5 and 7 A as well. A difference between otherwise identical samples before and after heat inactivation was recorded only with type 7 in these cases. The other types tested including type 7 A (S 1058) were not influenced in this way.

*Hedstrom C. E. & Lycke E.* Municipal Virological Laboratory and the Virological Laboratory of the Department of Bacteriology, University of Gothenburg, Gothenburg. AN EXPERIMENTAL STUDY OF OYSTERS AS VIRUS CARRIERS

Epidemics of hepatitis spread by raw oysters have been described from Sweden (Roos 1956 and Christensen 1956) and from U.S.A. (Mason and McLean 1961). Two mechanisms for the spread of virus with oysters have been postulated: one that the virus multiplies in the oysters; the second that the oysters only are passive carriers.

The second possibility was studied experimentally using poliovirus type 3 as a model strain. Oysters were stored alive in aquariums with sea water at 23°C. Samples of oysters and water were drawn after different time intervals and the concentration of virus in samples was titrated in monkey kidney cell cultures.

After a short time of exposure, less than two hours, poliovirus could be found in the oysters in the same concentration as that of the surrounding water. No concentration of virus above that level could be demonstrated. The virus was located in the soft tissue of the oysters. Infective virus was found in the oysters for a considerable long period of time after that the virus in the water had been removed or had become inactivated. Suspensions of homogenized oysters had a protective capacity against the inactivation of virus.

*Holm S. & Lycke F.* Municipal Virological Laboratory and the Department of Bacteriology, University of Gothenburg, Gothenburg. STUDIES ON VACCINIA LS ANTIGEN

The vaccinia LS antigen described by Craigie and Wishart (1956) is considered to be a protein molecule with two antigenically active sites (Shedlowsky and Smadel 1962). One of these, the L factor, is heat labile while the S factor is heat stable but is degraded by treatment with trypsin. The L factor is relatively resistant to trypsinization.

In the present studies the LS antigen was analyzed by means of double diffusion in gel technique. Four different antigen preparations were made. One was a homogenized material of vaccinia virus infected chorioallantoic membranes of chick embryos. The second was prepared similarly but was obtained from infected HeLa cell cultures. From these crude antigen preparations LS antigens were purified by means of differential centrifugation and isoelectrical precipitation.

Antisera were prepared by vaccination of rabbits with purified vaccinia virus material or by subcutaneous injections with the LS antigens. When the two crude preparations were tested against rabbit anti-vaccinia sera, five vaccinia-specific immunological systems could be demonstrated. One of the five immunological systems found was related to LS anti-LS. The two types of LS antigen preparations were found to be immunologically identical.

After the appropriate treatment for degradation of the L or the S components, the LS antigen reacted immunologically as one molecule with two different antigenic active sites.

body levels against types 1 and 3 30 per cent of the vaccinees tested had no demonstrable antibodies against the former and 40 per cent against the latter type Antibodies against type 2 were still present in 100 per cent

A group of patients who in 1957 had their primary two shots with inactivated vaccine and their booster with oral live polio vaccine (only type 1) was compared with the above mentioned control study In the live vaccine group antibodies against type 1 could still be demonstrated in 1961 in 100 per cent with mean titres about 1/125

*Bengtsson S & Philipson I* Institute of Virology University of Uppsala Uppsala  
COUNTRY CURRENT DISTRIBUTION OF POLIOVIRUS TYPE 1

To be published in Virology 1963

*Ohlson Marianne* Virus Department Central Bacteriological Laboratory of Stockholm City ANTIGENIC COMPOSITION OF ENTEROVIRUSES IN IMMUNODIFFUSION

A microtechnique with punched holes in agar on microscopic slides was used Native polioantigens prepared from infectious tissue culture fluid showed two precipitating antigens D and C The concentration of C antigen was rather high Different ways of concentration (by cellogel differential centrifugation or polymer phase separation in polyethyleneglycol dextran sulphate) yielded the same proportions D/C Serial passages with end point diluted inocula did not reduce the C antigen content However, treatment with freon 113 reduced the C antigen more than the D antigen

ECHO 6 apparently had only one precipitating antigen

Immunization with native polio antigens in guinea pigs gave a rise in D and C antibodies Heated antigens (56° C for 30 min) induced C antibodies but to a varying degree also antibodies of D character although the heated antigens had very low infectivity titres (0.7/ml) Homologous D antibodies could be absorbed with native antigen C antibodies with native or heated antigens

Heterotypic reactions between polioviruses occurred after prolonged immunization Polio type 2 hyperimmune sera precipitated with polio type 1 D antigen polio type 1 sera with polio types 2 and 3 antigens (mainly C) neither precipitated with ECHO 11 Individual variation of antibody response was observed

*Svart Malmberg Gunvor* Department of Virology Central Bacteriological Laboratory of Stockholm City THERMOLABILE SERUM FACTOR INFLUENCING THE NEUTRALIZATION OF ADENOVIRUS

By the technique of *Kjellen et al* (Acta Paediat Stockh 46:164 1957) human sera have been tested for their capacity of inhibiting certain adenovirus types It was demonstrated that a number of sera inhibited adenovirus type 7 Gemen to a greater extent before than after inactivation at 56° F for 30 minutes The addition of fresh guinea pig serum to inactivated samples restored the inhibitory capacity to the level of the unheated sample in several but not all of the sera tested Nor did fresh human serum without effect per se on adenovirus type 7 substitute the factor lost by heat inactivation Decomplementation by means of absorption of the complement on to a precipitate also removed the inhibitory capacity In this case however it was possible—by the addition of guinea pig serum partially and by the addition of

sheep erythrocytes. The haemolysis titre was estimated five days later. The cell material to be tested was also injected intraperitoneally.

It was first found that a homogenate of homologous spleen cells significantly increased the haemolysis response if given a few hours before or simultaneously with the antigen but not when the cell material was injected a few hours later. Thymocytes and liver cells were as effective as spleen cells. There was no significant difference between autologous and homologous cells in stimulating capacity. In the subsequent experiments the antigen and the homologous lymphoid material were given together.

Following the observation that whole cells and whole nuclei were not a requisite for the effect, further studies have been devoted to subcellular fractions (obtained by differential centrifugation of disintegrated spleen cells or thymocytes). Most of the activity seemed to reside in the nuclei. Microsomes and the 10,000 g supernatant (50 min) had no significant effect. Incubation of thymocyte nuclei with DNase deprived them of the stimulating effect. A significant amount of the activity was released from the nuclei during incubation.

*Laurit Anna Brilla Institute of Bacteriology, University of Lund, Lund*  
 (COMPARISON OF THE AGGLUTINATION OF E<sub>1</sub> AND F<sub>112</sub> CELLS BY  
 RHEUMATOID ARTHRITIC SERA)

Rheumatoid arthritis sera were tested on their ability to agglutinate sheep red cells (sensitized by rabbit anti sheep red cell antibodies). E<sub>1</sub> and such cells complexed with the complement factors C<sub>1</sub>, C<sub>4</sub> and C<sub>2</sub> of a human serum pool. F<sub>112</sub> and E<sub>112</sub> cells were in contrast to E<sub>1</sub> cells inagglutinable by rheumatoid arthritic sera. Further investigation showed that F<sub>112</sub> blood cells agglutinated to the same extent as E<sub>1</sub> cells did while all blood cell preparations carrying the activity of C<sub>4</sub>, E<sub>112</sub>, F<sub>112</sub>, F<sub>11</sub> and F<sub>1</sub> did not agglutinate. Haemolytically active C<sub>2</sub> on the sensitized cells did not seem to inhibit the agglutination.

On the other hand a rat anti rabbit  $\gamma$  globulin serum agglutinated E<sub>1</sub> and E<sub>112</sub> red cells to the same degree indicating that different antigenic determinants of the rabbit antibody react with rheumatoid arthritic factor and the anti rabbit  $\gamma$  globulin antibodies respectively.

*Astrid B. Central Laboratory for Clinical Bacteriology, Karolinska sjukhuset, Stockholm*  
 PHAGOCYTOSIS IN RHEUMATOID ARTHRITIS

Kilbäck *et al.* (1957) showed that  $\beta$  haemolysing streptococci engulfed in granulocytes in peripheral blood from healthy individuals survived intracellularly for less than 20 minutes.

Ollhagen has found (to be published) that it often is difficult to treat successfully infections with  $\beta$  haemolysing streptococci in patients with rheumatoid arthritis. It has therefore seemed of interest to study the intracellular survival time of streptococci engulfed in phagocytes from patients with rheumatoid arthritis. A slight modification of the method of Wilson *et al.* has been used.

In experiments on 24 healthy individuals the results were in agreement with those of Wilson *et al.*

26 experiments were made with granulocytes from patients with rheumatoid arthritis. In 8 of them the streptococci were still viable after having been engulfed for 30 minutes. As a proof of viability was taken the ability of the engulfed streptococci to manifest multiplication after disruption of the granulocyte.



*Hermansson S* Institute of Virology University of Uppsala Uppsala INHIBITION OF INTERFERON IN DOUBLY INFECTED CELLS

Inoculation of large amounts of Newcastle disease virus (NDV) into calf kidney cultures resulted as a rule in a slight virus multiplication probably due to the presence of an interferon like substance in the inoculated virus fluid. The auto-interfering activity of NDV however could be inhibited if the cultures prior to the inoculation of NDV was infected with parainfluenza 3 virus (PI-3). An infection with PI-3 could also activate the reproduction of NDV in cultures which had been persistently infected with the latter virus. A possible explanation to this phenomenon was that NDV induced to the production of less amounts of interferon in cultures infected with PI-3 than in normal cell cultures and that an infection with PI-3 suppressed the antiviral action of interferon on the multiplication of NDV.

*Strannegård B & Lycke F* Municipal Virological Laboratory and the Virological Laboratory of the Department of Bacteriology University of Gothenburg Gothenburg STUDIES ON ANTIGEN ANTIBODY SYSTEMS WITH REFERENCE TO MONONUCLEOSIS INFECTION

Sera from patients with mononucleosis infection contain ox cell haemolysins and agglutinins directed against ox and sheep cells. In the present study the ox cell haemolysin (OCH) titres were found to be well correlated to titres obtained in sheep cell agglutination tests (PB tests). The ox cell agglutinins seemed to be identical with the ox cell haemolysins. Absorption studies indicated that two or possibly three types of antibodies are reacting in the OCH and PB tests.

Antigenic preparations obtained from heated and mechanically disintegrated ox and sheep erythrocytes gave rise to precipitation lines when allowed to react with sera with high OCH and PB titres. The occurrence of precipitins was found to be correlated to the concentrations of ox cell haemolysins and sheep cell agglutinins. The diffusion in gel studies demonstrated two antigenic factors in ox erythrocytes. One of these factors was related to the only one demonstrated in sheep erythrocytes. These findings were supported by the absorption studies.

The antibodies precipitating ox cell antigens appeared to be  $\beta$   $\gamma$  globulins.

*Fjellström K E* Department of Clinical Chemistry University Hospital Uppsala ELECTROPHORETIC STUDIES ON HUMAN COMPLEMENT LOCALIZATION OF C FACTORS IN STARCH GEL

To be published in Acta path et microbiol scandinav 1963

*Akländer J & Hogman C* Department of Clinical Chemistry and the Transfusion Service University Hospital Uppsala FRACTIONATION OF HUMAN BLOOD GROUP ANTIBODIES BY GEL FILTRATION

To be published in Scand J Lab Clin Invest 1963

*Berglund K, Fagraeus Astrid & Skellö O* Rheumatology Department Karolinska sjukhuset National Bacteriological Laboratory Stockholm and the Institute of Medical Chemistry University of Uppsala Uppsala THE ENHANCING EFFECT OF LYMPHOID CELLS AND CELL FRACTIONS ON ANTIBODY RESPONSE

The test object has been the early phase of the primary haemolysin response in the rat. The animals were given the intraperitoneal injection of a small amount of

sheep erythrocytes. The haemolysis titre was estimated five days later. The cell material to be tested was also injected intraperitoneally.

It was first found that a homogenate of homologous spleen cells significantly increased the haemolysis response if given a few hours before or simultaneously with the antigen but not when the cell material was injected a few hours later. Thymocytes and liver cells were as effective as spleen cells. There was no significant difference between autologous and homologous cells in stimulating capacity. In the subsequent experiments the antigen and the homologous lymphoid material were given together.

Following the observation that whole cells and whole nuclei were not a requisite for the effect, further studies have been devoted to subcellular fractions (obtained by differential centrifugation of desintegrated spleen cells or thymocytes). Most of the activity seemed to reside in the nuclei. Microsomes and the 105 000 g supernatant (30 min) had no significant effect. Incubation of thymocyte nuclei with DNase deprived them of the stimulating effect. A significant amount of the activity was released from the nuclei during incubation.

*Lurell Anna Britta* Institute of Bacteriology, University of Lund, Lund  
(COMPARISON OF THE AGGLUTINATION OF F4 AND E4<sub>142</sub> CELLS BY  
RHEUMATOID ARTHRITIC SERA)

Rheumatoid arthritis sera were tested on their ability to agglutinate sheep red cells (sensitized by rabbit anti sheep red cell antibodies). F4 and such cells complexed with the complement factors C1, C4 and C2 of a human serum pool. E4<sub>142</sub>, F4<sub>11</sub> cells were in contrast to F4 cells inagglutinable by rheumatoid arthritic sera. Further investigation showed that F4<sub>11</sub> blood cells agglutinated to the same extent as F4 cells did while all blood cell preparations carrying the activity of C4, E4<sub>142</sub>, F4<sub>11</sub>, F4<sub>16</sub> and E4<sub>1</sub> did not agglutinate. Haemolytically active C2 on the sensitized cells did not seem to inhibit the agglutination.

On the other hand a rat anti rabbit  $\gamma$  globulin serum agglutinated E4 and E4<sub>142</sub> red cells to the same degree indicating that different antigenic determinants of the rabbit antibody react with rheumatoid arthritic factor and the anti rabbit  $\gamma$  globulin antibodies respectively.

*Wiström H.* Central Laboratory for Clinical Bacteriology, Karolinska sjukhuset, Stockholm. PHAGOCYTOSIS IN RHEUMATOID ARTHRITIS

Wiström *et al.* (J. Exp. Med. 106: 777, 1957) showed that  $\beta$  haemolysing streptococci engulfed in granulocytes in peripheral blood from healthy individuals survived intracellularly for less than 20 minutes.

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In experiments on 24 healthy individuals the results were in agreement with those of Wiström *et al.*

26 experiments were made with granulocytes from patients with rheumatoid arthritis. In 8 of them the streptococci were still viable after having been engulfed for 30 minutes. As a proof of viability was taken the ability of the engulfed streptococci to manifest multiplication after disruption of the granulocyte.

*Hermolsson S* Institute of Virology, University of Uppsala Uppsala INHIBITION OF INTERFERON IN DOUBLY INFECTED CELLS

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*Strannegård Ö & Tjelle F* Municipal Virological Laboratory and the Virological Laboratory of the Department of Bacteriology, University of Gothenburg Gothenburg STUDIES ON ANTIGEN ANTIBODY SYSTEMS WITH REFERENCE TO MONONUCLEOSIS INFECTION

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Antigenic preparations obtained from heated and mechanically disintegrated ox and sheep erythrocytes gave rise to precipitation lines when allowed to react with sera with high OCH and PB titres. The occurrence of precipitins was found to be correlated to the concentrations of ox cell haemolysins and sheep cell agglutinins. The diffusion in gel studies demonstrated two antigenic factors in ox erythrocytes. One of these factors was related to the only one demonstrated in sheep erythrocytes. These findings were supported by the absorption studies.

The antibodies precipitating ox cell antigens appeared to be  $\beta$  M globulins.

*Fjellström K F* Department of Clinical Chemistry, University Hospital Uppsala ELECTROPHORETIC STUDIES ON HUMAN COMPLEMENT LOCALIZATION OF C' FACTORS IN STARCH GEL

To be published in Acta path et microbiol scandinav 1963

*Källander J & Högman C* Department of Clinical Chemistry and the Transfusion Service, University Hospital Uppsala FRACTIONATION OF HUMAN BLOOD GROUP ANTIBODIES BY GEL FILTRATION

To be published in Scand J Lab Clin Invest 1963

*Berglund K, Lagercrantz Astrid & Sköld H* Rheumatology Department Karolinska sjukhuset National Bacteriological Laboratory Stockholm and the Institute of Medical Chemistry, University of Uppsala Uppsala THE ENHANCING EFFECT OF LYMPHOID CELLS AND CELL FRACTIONS ON ANTIBODY RESPONSE

The test object has been the early phase of the primary haemolysin response in the rat. The animals were given one intraperitoneal injection of a small amount of

*Lindberg A & Kallings L B* National Bacteriological Laboratory Stockholm  
 ROLE OF LYSOGENICITY IN O-1 RESISTANT SALMONELLA BACTERIA

The bacteriophage O 1 acting on most *Salmonella* bacteria separates the *Salmonella* strains into two groups: strains sensitive to low phage concentrations and supporting the propagation of new particles and strains only influenced by high concentrations and unable to produce phages (Kallings 1961). The purpose of this paper is to study the cause of the different sensitivity to the phage. As the resistance was assumed to be due to latent phages blocking the synthesis of O-1 phages, experiments are reported concerning the isolation of latent phages and attempts to transform sensitive strains to resistant ones by means of these phages.

Twenty *Salmonella* strains were chosen and tested for O-1 phage activity. One of the two resistant strains was induced by exposure to ultraviolet radiation; the supernate was then tested against the other strains by spot technique. A temperate phage (105) was isolated and found able to transform the sensitive *S. typhimurium* strains to resistant ones. The O-1 resistant cultures were induced to prove that they were lysogenized by phage 105. The supernates exhibited the same lytic spectrum as phage 105. Their activity was neutralized by hyperimmune serum against phage 105.

Phages with the same ability to change the sensitivity to phage O-1 were isolated from other cultures belonging to different *salmonella* groups.

*Danielsson D & Laurell G* Institute of Bacteriology, University of Uppsala  
 UPPSALA RAPID DETECTION OF SMALL NUMBERS OF BACTERIA IN WATER  
 BY MEANS OF FLUORESCENT ANTIBODIES

Fluorescent antibody technique combined with membrane filter technique has been used to study bacterial contamination of water. Known concentrations of the test bacteria, enteropathogenic *Esch. coli*, were added to fixed volumes of water. As a rule, 1 litre was filtered through a membrane filter (Millipore HAWG 47 mμ). At bacterial densities of 500-1000 bacteria/litre, identification was accomplished in two hours. At lower concentrations, the membrane filters were incubated for varying periods in broth and subsequently centrifuged to concentrate the bacteria. By this technique, a bacterial concentration of 50 bact./litre could be detected in 6 hours, 15-20 bact./litre in 8-10 hours and 2-5 bact./litre in 12-18 hours respectively. The technique was also quantitative at different ratios (0.2-100) between concentration of contaminating bacteria and test bacteria. By conventional bacteriological and serological diagnosis, could be made after 48 hours at the earliest and at a high ratio of contaminating bacteria to test bacteria, the latter could often not be isolated.

Preliminary attempts have been made to detect bacteria directly on nonfluorescent membrane filters (Millipore HAWG) by the use of fluorescent antibodies. This method offers a chance of further reducing the time.

In a 6 months field study, samples of the water of a river in central Sweden (the Färis) were examined as to the occurrence of enteropathogenic *Esch. coli*. By the use of fluorescent antibodies, 10 different strains of these bacteria were detected. 6 of these were also isolated by conventional tests.

The experimental conditions are too uncertain to allow any positive conclusions to be drawn. Further experiments are planned with a technique that allows quantitative estimation of engulfment and cytopexis.

*Hedersstedt B* : National Bacteriological Laboratory, Stockholm, Sweden  
**TRIPONEMA PALLIDUM IMMOBILIZATION IN NORMAL SERUM**

*Treponema pallidum* immobilization is demonstrable in unheated normal serum from man and certain animals.

Aliquots of unheated serum and treponemes, suspended in a modified (e.g. reducing SH compounds replaced by aq. dest.) Nelson medium were mixed and incubated at 35° C in (O<sub>2</sub>-N<sub>2</sub>) atmosphere for 90 minutes.

The immobilizing activity was found in the majority of healthy persons studied though in low dilutions only ( $\leq 1:16$ ). This serum activity occurred in rat, sheep and ape, but not in guinea pig, rabbit or hamster.

The immobilizing serum activity disappeared after heat-inactivation or complementation with specific precipitate. No activity was found in R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> or R<sub>4</sub> serum. Zymosan treatment at 17° C did not abolish the immobilizing activity. This activity was lost after bentonite treatment and was restored by egg white lysozym. There was no quantitative correlation between immobilizing effect and complement or lysozym activity in different sera.

The non specific immobilizing activity did not seem to affect the result of the specific TPI test, probably due to the use in this test of heated human serum and an inhibiting effect of guinea pig and rabbit sera on the non specific immobilizing activity.

*Lindbom G* : Institute of Bacteriology, University of Uppsala, Uppsala, THE  
**GENESIS AND INTRAMURAL SPREAD OF A STAPHYLOCOCCAL EPIDEMIC**

During 1961 the University Hospital in Uppsala was troubled by a series of infections where *Staph. aureus* phage type 75/77 was isolated. This strain had been uncommon in the preceding years.

The first cases were noticed in March when patients undergoing thoracic surgery developed postoperative wound infections caused by highly antibiotic resistant staphylococci. After 3 months 8 patients, 2/3 of whom had been tracheotomized had been infected. Circumstantial evidence seemed to indicate the postoperative unit as major source of infection.

Retrospective analysis of epidemiological data from the whole hospital strongly suggested that the 75/77 strains were brought to Uppsala from a regional hospital by a few patients already infected and sent here for specialized treatment. Portals of entry were the departments of orthopedics, plastic and thoracic surgery. The strain was dispersed to other parts of the hospital by patients transferred for diagnostic and/or therapeutic purposes. In June all major specialities were affected by the epidemic. Finally the strain was spread to local hospitals by patients returning for further treatment.

All in all 47 patients infected by this strain could be traced. It was an important factor in the death of 8 patients.

This epidemic demonstrated both the spread of staphylococcal disease from one hospital to another and within one hospital.



Kallings L O, Bucht H, Wehle B & Örsten P. National Bacteriological Laboratory and the IVth Medical Clinic (The Kidney Clinic), S:t Erik's Hospital, Stockholm.  
TREATMENT OF CHRONIC PYELONEPHRITIS WITH AMPICILLIN

Infections due to *Proteus mirabilis*, *Escherichia coli* and *Aerobacter aerogenes* in 18 cases of chronic pyelonephritis were treated with 0.75 to 4.0 g ampicillin orally daily during periods varying from some weeks to a year. Most cases were previously found to resist treatment with other drugs.

The sensitivity of the bacteria to ampicillin was tested and the ampicillin concentration in serum and urine determined. The treatment was followed clinically with special reference to the kidney function and by assay of the bacterial concentration in the urine. The influence on the intestinal flora was examined by cultivation and determination of stercobilin and bilirubin in faeces and of pyruvic acid in serum.

At the level of  $10^5$ – $10^6$  cells/ml the MIC in broth varied with the different bacterial strains from 3 to 12,500 µg/ml.

Clinical improvement occurred in most cases. Sterile urine was obtained in 6 cases and the bacterial counts were markedly reduced in 4 cases. Failures were obtained in patients infected with *aerogenes* or with the most resistant *proteus* bacteria and in those with renal calculi. The normal intestinal flora was temporarily depressed in a few cases. No complications occurred.

Tunell G & Frisk R. Central Bacteriological Laboratory of Stockholm City.  
STUDIES ON PENICILLINS OF THE PHENOXYMETHANT SERIFS

The compounds studied are phenoxy methyl, ethyl, propyl and isopropyl, butyl and isobutyl, amyl and hexylpenicillin. For comparison benzyl  $\alpha$ -amino benzyl and 2,6-dimethoxyphenylpenicillin were included.

The general *in vitro* activity against sensitive organisms decreased with increasing length of the side chain, though at most by two steps in a twofold dilution series. Against penicillinase-producing staphylococci an increase in activity from benzyl to phenoxyhexylpenicillin of the same magnitude was found. Iso forms were slightly less active than normal ones but more stable against penicillinase.

In triple crossover experiments in groups of ten patients the serum concentrations after single oral doses of 0.3 g were followed. Higher and more lasting concentrations were found the longer was the side chain. After 1 hour phenoxyhexylpenicillin gave 5 times the concentration of phenoxyethylpenicillin, after 6 hours as much as 15 times.

Winge Heden Kerstin. Central Laboratory of Clinical Bacteriology, Karolinska sjukhuset, Stockholm. STRERILIZATION BY ETHYLENE OXIDE WITHOUT SPECIAL EQUIPMENT

To be published in Acta path. et microbiol. scandinav. 1963

Marked glucosuria (lowest 1.5 and highest 2.9 g per 24 hours) and inhibited body growth were noted in 6 of the 8 rats in the alloxan group. The 2 remaining rats in this group did not show glucosuria, and hence were considered to have no manifest diabetes. As appears from the tables and from Figs 3 and 4, these 2 rats responded in the same way as the controls in all other respects as well. No change in the severity of diabetes, as reflected by the degree of glucosuria, could be demonstrated during the fortnight of ovarian hormone treatment.

TABLE I  
Weights of Adrenals and Thymus in Control and Alloxan Diabetic Rats

	No of rat	Body weight (g)			Adrenal weight (mg)	Thymus weight (mg)
		Before injections of		At autopsy		
		Alloxan	Ovarian hormones			
A Control rats	C1	110	170	183	53.8	160
	C2	118	190			
	C3	86	140	154	40.4	104
	C4	105	170	183	53.9	198
	C5	89	124	147	42.9	215
	C6	95	130	145	58.5	206
	C7	87	130	155	43.5	289
	C8	85	125	137	31.5	145
	C9	77	125	148	38.7	218
	Mean weight $\pm$ standard error of the mean		91 $\pm$ 4	141 $\pm$ 7	157 $\pm$ 7	45.4 $\pm$ 3.2
B Rats injected with alloxan. Manifest diabetes	Ax2	105	139	156	48.5	113
	Ax3	87	95	119	44.5	122
	Ax4	111	100	118	48.9	81
	Ax5	96	122	129	60.3	74
	Ax6	71	78	87	52.4	22
	Ax8	90	110	120	49.5	68
	Mean weight $\pm$ standard error of the mean		89 $\pm$ 5	107 $\pm$ 9	121 $\pm$ 10	50.7 $\pm$ 2.2
C Rats injected with alloxan. No manifest diabetes	Ax1	119	182	191	51.7	149
	Ax7	117	149	145	47.6	178

At autopsy the diabetic rats had a body weight significantly lower than the controls ( $P < 0.01$ ). The absolute weight of the adrenals in the former was higher but not significantly so ( $P = 0.2$ ). In relation to body weight the diabetic rats apparently had a higher adrenal weight than the controls. The lower thymus weight in the diabetic rats ( $P < 0.01$ ) seems only partly explained by the body weight difference.



## MATERIAL AND METHODS

The experiments were made on a group of 17 male rats of a Wistar strain maintained for many years at the Department of Pathology. Born 1 March 1961 the rats were castrated between the 19th and 28th day of age, when they weighed 30 to 40 g. From 21 to 31 days after being castrated 8 of the rats received a subcutaneous injection of 0.15 mg alloxan per g body weight. The remaining 9 rats were used as controls. When 21 days had elapsed after the alloxan injection the 3rd right thoracic mammary gland was extirpated for whole mount preparation both from experimental rats and controls. During the next 14 days, all the rats were given daily intramuscular injections of 10  $\mu$ g oestrone (100  $\mu$ g/ml in arachis oil) and 4 mg progesterone ("Progestin" brand Pharmacia 25 mg per ml). The day after the last injection the rats were exsanguinated under ether, the adrenals and thymus being excised and weighed. The 3rd left thoracic and the left abdominal mammary glands were excised for preparation of whole mounts. These specimens were prepared and stained with gallocyanine chromalum as described previously (Åhrén & Etienne 1957, Åhrén 1959 b). For histological examination the corresponding right abdominal mammary gland was excised, fixed in Bouin's solution, embedded in paraffin and cut into 5  $\mu$  sections which were stained with haematoxylin eosine and according to Weigert van Gieson.

All the rats were given tap water and a special, commercial rat bread *ad libitum* throughout the experiment. All were weighed at regular intervals of about a week. The degree of glucosuria (in g per 24 hours) was estimated by Benedict's quantitative method. During periods when the urinary glucose output was estimated the rats were transferred to separated cages adapted especially for urine collection. Some contamination of the urine by food was unavoidable.

The whole mount mammary gland preparations were examined microscopically in order to assess the type and degree of glandular development. In so doing we adopted a scoring system slightly modified in accordance with the below definitions introduced by Cowie & Polley (1947) and used over a period of years by one of us (e.g. Åhrén 1959 b).

*Duct System Development*

- + Restricted duct system showing little branching
- ++ Moderately extensive duct system showing moderate branching
- +++ Widespread duct system showing profuse branching

*Club-Shaped End Buds*

- + Few end buds present
- ++ Numerous end buds irregularly disposed around the periphery
- +++ Abundant end buds regularly disposed around the periphery

*Alveolar Development*

- + Few alveoli present
- ++ Moderate alveolar development, evenly distributed
- +++ Ducts covered with alveoli

In the column for alveolar development in the table the score '(+)' will also be found. This score was applied to glands exhibiting a few indeterminate formations which however might have been alveoli. For as shown by Richardson (1947) to distinguish clusters of small ducts from true alveoli may be very difficult or even impossible.

## RESULTS

Table 1 gives body weights at different times during the experimental period and weights of the adrenals and thymus at autopsy. Table 2 gives a synopsis of mammary gland development before and after treatment with ovarian hormones. Figs 1-10 illustrate typical whole mount preparations and paraffin sections of mammary glands from alloxan diabetic rats and controls.

## PLATE I



3



2

4

TABLE 2

*Synopsis of Mammary Gland Development before and after Treatment with Ovarian Hormones in Control and Alloxan Diabetic Rats*

	No of rat	Mammary glands					
		Duct system		End buds		Alveoli	
		Before ovarian hormones	After ovarian hormones	Before ovarian hormones	After ovarian hormones	Before ovarian hormones	After ovarian hormones
A Control rats	C1	++	+++	+	++	0	+++
	C2	++	+++	0	+++	(+)	+++
	C3	++	+++	+	++	0	+++
	C4	++	+++	++	++	0	+++
	C5	++	+++	0	+	0	++
	C6	++	+++	++	++	0	+++
	C7	++	+++	0	++	0	++
	C8	++	+++	+	++	0	++
B Rats injected with alloxan Manifest diabetes	Ax2	+	++	0	+++	(+)	+
	Ax3	+	++	0	++	(+)	+
	Ax4	+	++	0	+++	(+)	+
	Ax5	+	++	0	++	(+)	+
	Ax6	+	++	0	++	0	+
	Ax8	+	++	0	++	(+)	+
C Rats injected with alloxan No manifest diabetes	Ax7	++	+++	++	+	0	+++
	Ax1	++	+++	+	++	(+)	+++

*(Comments to Plates I-III)*

Figs 1-8 show whole mount preparations of mammary glands stained with gallo cyanin chromalum and photographed at the same magnification (see Plate II). Figs 9 and 10 show microphotographs of 5  $\mu$  thick sections through mammary glands stained with Weigert van Gieson. The microphotographs were taken at the same magnification ( $\times 110$ ).

*Plate I*

- Fig 1* (Table 1 C 6) Castrated male rat. Third right thoracic gland extirpated before the injections of ovarian hormones—Small gland. No alveoli. Many small end buds.
- Fig 2* Same rat as in Fig 1. Third left thoracic gland after 14 days of treatment with 10  $\mu$ g oestrone and 4 mg progesterone daily—This gland covered a very large area and the photograph shows approximately one fourth of the gland—The duct system is markedly developed and there is an extensive lobule alveolar development.
- Fig 3* (Table 1 Ax 7) Castrated male rat injected with alloxan but without manifest diabetes. Third right thoracic gland extirpated before the injections of ovarian hormones—Small gland. No alveoli. Many small end buds.
- Fig 4* Same rat as in Fig 3. Third left thoracic gland after 14 days of treatment with 10  $\mu$ g oestrone and 4 mg progesterone daily. The photograph shows approximately one fourth of the gland—Marked development of the duct system and extensive lobule alveolar development.



3

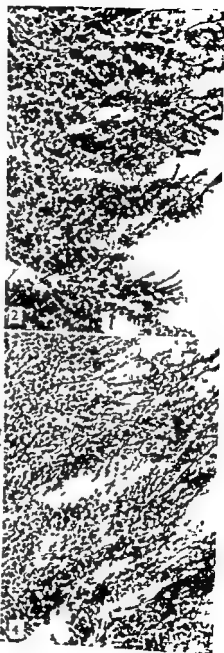


TABLE 2

*Synopsis of Mammary Gland Development before and after Treatment with Ovarian Hormones in Control and Alloxan Diabetic Rats*

	No of rat	Mammary glands					
		Duct system		End buds		Alveoli	
		Before ovarian hormones	After ovarian hormones	Before ovarian hormones	After ovarian hormones	Before ovarian hormones	After ovarian hormones
A Control rats	C1	++	++++	+	++	0	+++
	C2	++	++++	0	+++	(+)	+++
	C3	++	++++	+	++	0	+++
	C4	++	++++	++	++	0	+++
	C5	++	++++	0	+	0	++
	C6	++	++++	++	++	0	+++
	C7	++	++++	0	++	0	++
	C9	++	++++	+	++	0	++
	C8	++	++++	+	++	0	++
B Rats injected with alloxan Manifest diabetes	Ax2	+	++	0	+++	(+)	+
	Ax3	+	++	0	++	(+)	+
	Ax4	+	++	0	+++	(+)	+
	Ax5	+	++	0	++	(+)	+
	Ax6	+	++	0	++	0	+
	Ax8	+	++	0	++	(+)	+
C Rats injected with alloxan No manifest diabetes	Ax7	++	+++	++	+	0	+++
	Ax1	++	+++	+	++	(+)	+++

### *Comments to Plates I-III*

Figs 1-8 show whole mount preparations of mammary glands stained with gallo cyanin chromalum and photographed at the same magnification (see Plate II). Figs 9 and 10 show microphotographs of 5  $\mu$  thick sections through mammary glands stained with Weigert van Gieson. The microphotographs were taken at the same magnification ( $\times 110$ ).

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- Fig 3* (Table 1 Ax 7) Castrated male rat injected with alloxan but without manifest diabetes Third right thoracic gland extirpated before the injections of ovarian hormones—Small gland No alveoli Many small end buds
- Fig 4* Same rat as in Fig 3 Third left thoracic gland after 14 days of treatment with 10  $\mu$ g oestrone and 4 mg progesterone daily The photograph shows approximately one fourth of the gland—Marked development of the duct system and extensive lobule alveolar development

## *Mammary Gland Development*

### *A Before Administration of Ovarian Hormones*

All of the diabetic rats had a duct system which was less developed than that in the controls (Table 2 and Figs 1, 3, 5 and 7). In addition most of the controls exhibited club shaped end buds (Figs 1 and 3), but none of the diabetic rats had such end buds (Table 2, Figs 5 and 7). In the controls alveoli were absent in the mammary glands. The mammary glands in the diabetic rats lacked distinct alveoli but most of these animals presented some small formations which could have been either clusters of alveoli or groups of small ducts.

### *B After Administration of Ovarian Hormones*

Table III and Figs 2, 4, 6 and 8 reveal that during the period of hormone treatment the duct system had developed markedly both in diabetic rats and controls. Nevertheless, the controls still displayed better duct system development and greater glandular area than the diabetic rats. All mammary glands from both groups exhibited numerous club-shaped end buds (Figs 2, 4, 6 and 8), the degree of end-bud development in the diabetic group being at least as high as in the control group.

All mammary glands from the controls showed pronounced alveolar development and in the majority of these glands the entire duct system was covered with groups of alveoli (Figs 2 and 4). The mammary glands from the diabetic rats showed a clear but slight alveolar development (Figs 6 and 8) which in all cases was less marked than in the controls.

Mammary gland paraffin sections from the controls exhibited a multiplicity of large groups of small alveoli in which the lumen, if present, was small (Figs 9). In the glands from the diabetic rats the alveolar groups were much fewer (Fig 10). The alveolar epithelium in the latter group was somewhat higher than that in the controls, and,

### *Plate II*

- Fig 5** (Table 7 Ax 2) Castrated male rat injected with alloxan and with manifest diabetes. Third right thoracic gland extirpated before the injections of ovarian hormones—Very restricted duct system. No end buds. Eventually a few small groups of alveoli.
- Fig 6** Same rat as in Fig 5. Third left thoracic gland after 14 days of treatment with 10  $\mu$ g oestrone and 4 mg progesterone daily—Marked growth of the duct system. Large club shaped end buds. Very slight alveolar development in the central part of the gland.
- Fig 7** (Table 1 Ax 4) Castrated male rat injected with alloxan and with manifest diabetes. Third right thoracic gland extirpated before the injections of ovarian hormones—Very restricted duct system. No end buds. Eventually a few small groups of alveoli in one part of the gland.
- Fig 8** Same rat as in Fig 7. Third left thoracic gland after 14 days of treatment with 10  $\mu$ g oestrone and 4 mg progesterone daily—Marked growth of the duct system. Many large end buds. Slight alveolar development in the central part of the gland.



5



6



7



8

5 mm



in addition, the alveolar lumen in the diabetic rats was larger than in the controls

### DISCUSSION

The present investigation has demonstrated that ovarian hormones can stimulate an extensive ductal growth and a slight but clear alveolar development in the mammary glands of alloxan diabetic rats. This result shows that a normal insulin secretion from the islet  $\beta$  cells is not a prerequisite for the stimulation of mammary gland growth by ovarian hormones in the rat. This result, however, does not prove that the ovarian hormones *in vivo* can stimulate growth processes within the mammary glands in complete absence of insulin, because an alloxan diabetic rat cannot be considered as an "insulin free" animal.

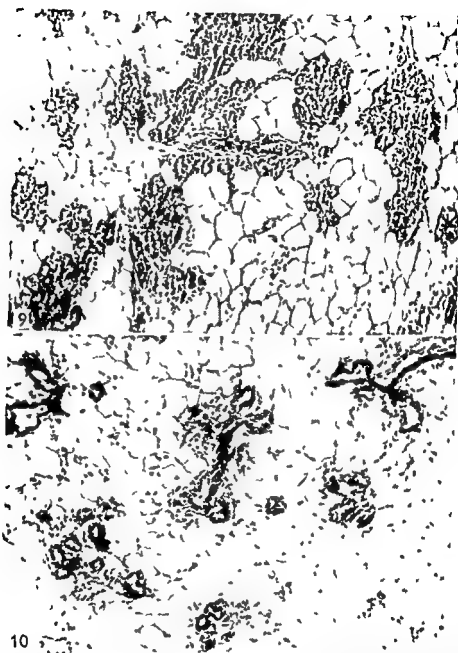
After administration of ovarian hormones the duct system of the mammary glands in the two groups of rats showed approximately the same growth increment although the duct system was less developed in the diabetic rats both before and after administration of the ovarian hormones. Many observations (*e.g.* Lyons *et al.* 1955, 1958; Ahren 1959a) indicate that the presence of somatotrophic hormone is required if the ovarian hormones are to be capable of stimulating a more marked duct growth in the mammary glands of the rat. Hence the ability of the pituitary to elaborate somatotrophic hormone does not seem to be significantly impaired in the alloxan diabetic rat and, judging by the well developed duct system in their mammary glands, it could very possibly be normal or even increased. Published reports on the production of somatotrophic hormone in alloxan diabetic rats are conflicting. Thus it has been reported that in such rats the somatotrophic hormone activity of plasma is reduced (Lawrence *et al.* 1958), whilst that of the pituitary is increased (Solomon 1958).

The degree of alveolar development after steroid treatment was less pronounced in the alloxan diabetic rats than in the controls. This might indicate that ovarian hormones are unable to induce normal alveolar growth in the mammary glands unless insulin is present in sufficient amounts, implying that insulin deficiency was directly responsible for the inhibited alveolar development in the alloxan diabetic rats. Considering that ovarian hormones are unable to promote alveolar growth in the mammary glands of the rat unless prolactin is present (*e.g.* Lyons *et al.* 1955, 1958; Ahren 1959) another possibility to explain the poor alveolar development in the alloxan diabetic rats is that inadequate prolactin production rather than insulin deficiency was the direct cause of the alveolar underdevelopment. Many observations suggest that the secretion of FSH and LH is impaired in diabetic animals (*e.g.* Lawrence & Contopoulou 1960a, b; Hunt & Baily 1961). Whether such animals also have reduced prolactin secretion has apparently not been studied but it seems far from improbable that such is the case.

Another point worth considering is the fact that the production of



## PLATE III



*Fig 9* (Table 1 C 7) Castrated male rat—Right abdominal gland after 14 days of treatment with 10  $\mu$ g oestrone and 4 mg progesterone daily—Many large groups of alveoli

*Fig 10* (Table 1 A x 2) Castrated male rat injected with alloxan and with manifest diabetes—Right abdominal gland after 14 days of treatment with 10  $\mu$ g oestrone and 4 mg progesterone daily—Very few but somewhat more open alveoli than in the glands from the control rats (compare Fig 9)

in addition, the alveolar lumen in the diabetic rats was larger than in the controls

### DISCUSSION

The present investigation has demonstrated that ovarian hormones can stimulate an extensive ductal growth and a slight but clear alveolar development in the mammary glands of alloxan diabetic rats. This result shows that a normal insulin secretion from the islet  $\beta$  cells is not a prerequisite for the stimulation of mammary gland growth by ovarian hormones in the rat. This result, however, does not prove that the ovarian hormones *in vivo* can stimulate growth processes within the mammary glands in complete absence of insulin because an alloxan diabetic rat cannot be considered as an insulin free animal.

After administration of ovarian hormones the duct system of the mammary glands in the two groups of rats showed approximately the same growth increment although the duct system was less developed in the diabetic rats both before and after administration of the ovarian hormones. Many observations (*e.g.* Lyons *et al.* 1955, 1958; Ahren 1959) indicate that the presence of somatotrophic hormone is required if the ovarian hormones are to be capable of stimulating a more marked duct growth in the mammary glands of the rat. Hence the ability of the pituitary to elaborate somatotrophic hormone does not seem to be significantly impaired in the alloxan diabetic rat and, judging by the well developed duct system in their mammary glands it follows

that the secretion of somatotrophic hormone is not reduced (Lawrence *et al.* 1958), whilst that of the pituitary is increased (Solomon 1958).

The degree of alveolar development after steroid treatment was less pronounced in the alloxan diabetic rats than in the controls. This might indicate that ovarian hormones are unable to induce normal alveolar growth in the mammary glands unless insulin is present in sufficient amounts, implying that insulin deficiency was directly responsible for the inhibited alveolar development in the alloxan diabetic rats. Considering that ovarian hormones are unable to promote alveolar growth in the mammary glands of the rat unless prolactin is present (*e.g.* Lyons *et al.* 1955, 1958; Ahren 1959) another possibility to explain the poor alveolar development in the alloxan diabetic rats is that inadequate prolactin production rather than insulin deficiency was the direct cause of the alveolar underdevelopment. Many observations suggest that the secretion of FSH and LH is impaired in diabetic animals (*e.g.* Lawrence & Contopoulos 1960 a, b; Hunt & Bailey 1961). Whether such animals also have reduced prolactin secretion has apparently not been studied but it seems far from improbable that such is the case.

Another point worth considering is the fact that the production of

corticosteroid apparently is enhanced in alloxan diabetic rats (cf Dury 1953, Weller & Bettge 1954, Kalant 1955, Angervall 1959). In our experiments adrenal hyperplasia combined with thymus atrophy are morphological signs of increased secretion of corticosteroids. It has been shown that some corticosteroids under certain experimental conditions can stimulate growth processes within the mammary glands (Selye 1954, Johnson & Meites 1955). Other studies indicate, however, that the same corticosteroids under other experimental conditions can inhibit such growth processes (Flux 1954, Ahren & Jacobsohn 1957). Therefore, it is impossible on the basis of the present experiments, to draw any conclusions as to whether the increased production of corticosteroids may have influenced significantly the mammary glands of the alloxan diabetic rats.

### SUMMARY

Mammary gland development in castrated, alloxan diabetic rats has been assessed before and after treatment with ovarian hormones (10  $\mu$ g oestrone and 4 mg progesterone daily for 14 days). Before such treatment the mammary glands of the alloxan diabetic rats were less developed than those of corresponding controls. The ovarian hormones stimulated in the alloxan diabetic rats a marked growth of the mammary duct system and a slight but clear alveolar development. These findings are discussed in the light of our present knowledge about the production in the alloxan diabetic rats of such hormones as have been shown to exert a significant growth promoting action on the mammary glands of the rat.

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## MAMMARY GLAND DEVELOPMENT IN NEWBORN OFFSPRING OF ALLOXAN DIABETIC RATS

By

K. ÅHREN and L. ÅNGERWALL

Received 13 x 62

In the pregnant rat diabetes seems to imply hormonal disturbances which can both enhance and inhibit foetal growth. It is imperfectly understood why diabetic rats can give birth to large offspring. The hypothesis has been advanced that maternal pituitary growth hormone (STH) is responsible for the enhanced growth. Increased production of growth promoting foetal insulin is another debated possibility. Moreover, the foetuses of non-insulinized alloxan diabetic rats are probably influenced by an increased production of maternal corticosteroids which might well inhibit foetal growth (Ångervall 1959).

Until puberty the development of the mammary glands in the rat parallels body growth (Astwood *et al* 1937, Cowie 1949). When the production of ovarian hormones in appreciable amounts begins during puberty, these hormones induce growth and differentiation of the mammary glands. However, the action of ovarian hormones on the mammary glands is influenced by STH and prolactin (*cf* Folley 1952). Under certain conditions growth processes in the mammary glands of the rat can also be affected by insulin (Åhren & Jacobsohn 1956, Åhren 1959). In addition some corticosteroids have been reported to be capable of stimulating the growth and development of the mammary glands (Selye 1954, Johnson & Meites 1955).

Accordingly, since the mammary gland is an effector organ for several of those hormones which appear to abnormally influence foetuses of alloxan diabetic rats, it should be interesting to study the mammary glands in these rats.

### MATERIALS AND METHODS

The experiments were done on male and female offspring of primiparous albino rats (Wistar strain)—25 young from 10 alloxan diabetic rats and 21 young from 6 normal rats. Diabetes was induced by a single subcutaneous injection of 0.15 mg per g rat of 5 per cent alloxan solution given on the 10th, 11th or 12th day of pregnancy. The rats were given a special rat bread and tap water *ad libitum*. Benedict's quantitative method was employed for estimating the degree of glucosuria. The alloxan diabetic rats had a urinary sugar output ranging from 2 to 4 g per 24

hours. As soon as the offspring had been born spontaneously they were weighed and decapitated. The weight of the 23 newborns in the diabetic group ranged between 3.76 and 6.97 g and that of the 21 newborns in the control group between 4.32 and 5.71 g.

The skin with the mammary glands of one half of the body was removed, stretched on tracing paper and fixed in Carnoy's solution for 12 to 24 hours. After dehydration with alcohol (1 hour each of absolute, 96 per cent, 80 per cent and 70 per cent alcohol) the 3rd thoracic mammary gland was excised under a dissection microscope, stained in gallocyanin-chromalum for 12 to 24 hours and mounted as a whole specimen on a glass slide.

The thoracic mammary glands of the other half of the body were rid of excess skin and fixed in 10 per cent formalin, embedded in paraffin and sliced into 5  $\mu$  sections which were stained with haematoxylin-eosine.

## RESULTS

**Whole specimens.** The 23 mammary glands from the offspring in the diabetic group showed primary, secondary and tertiary ducts radiating from the mammary region in a plane parallel to the surface of the skin (Figs 1 and 2). In 22 glands short side buds projected from these main ducts which generally were terminated with distinct, club shaped end buds. In 3 young from the same litter side-buds were lacking, although these too had distinct end buds. The mammary glands from the controls exhibited the same features in principle (Figs 3 and 4). Thus the mammary glands from the diabetic and control groups exhibited no significant qualitative or quantitative difference.

**Paraffin sections.** The main glandular ducts in this category were consistently open ended. They were demarcated by a fairly tall layer of cylindrical epithelium. Here and there outside this layer there was a layer of lower epithelial cells of various sizes. All epithelial cells exhibited a spheroid or ellipsoid nucleus which was moderately rich in chromatin and possessed a distinct nucleolus. Cells in mitosis were plenty. Corresponding to the side buds observed in the whole specimens there were small compact clumps of epithelium. The offspring in the diabetic and control groups exhibited no manifest differences.

## DISCUSSION

In the present investigation no manifest differences in mammary gland development were found between offspring of non insulinized, adult diabetic rats and control rats. The appearance of the glands showed good agreement with previously published data regarding the appearance of the mammary gland in newborn normal albino rats (Myers 1916, 1919; Astwood *et al.* 1937).

Some observations suggest that—unlike the case in adult rats—the foetal and early extrauterine development of the mammary glands is unaffected by ovarian hormones (Astwood *et al.* 1937). It has not been demonstrated whether foetal mammary gland development can be disturbed by other hormones which are capable of interfering with growth processes in mammary glands of the adult rat such as STH, prolactin

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Until puberty the development of the mammary glands in the rat parallels body growth (Astwood *et al.* 1937, Lowie 1949). When the production of ovarian hormones in appreciable amounts begins during puberty, these hormones induce growth and differentiation of the mammary glands. However, the action of ovarian hormones on the mammary glands is influenced by STH and prolactin (*e.g.* Folley 1952). Under certain conditions growth processes in the mammary glands of the rat can also be affected by insulin (Åhren & Jacobsohn 1956, Åhren 1959). In addition some corticosteroids have been reported to be capable of stimulating the growth and development of the mammary glands (Selye 1954, Johnson & Mettes 1955).

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The experiments were done on male and female offspring of primiparous albino rats (Wistar strain)—27 young from 10 alloxan diabetic rats and 21 young from 6 normal rats. Diabetes was induced by a single subcutaneous injection of 0.15 mg per 100 g rat of 5 per cent alloxan solution given on the 10th, 11th or 12th day of pregnancy. The rats were given a special rat bread and tap water *ad libitum*. Benedict's quantitative method was employed for estimating the degree of glucosuria. The alloxan diabetic rats had a urinary sugar output ranging from 2 to 4 g per 24

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Accordingly, since the mammary gland is an effector organ for several of those hormones which appear to abnormally influence foetuses of alloxan diabetic rats, it should be interesting to study the mammary glands in these rats.

### MATERIALS AND METHODS

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## Figs 1-4

show whole mount preparations of mammary glands stained with gallicyanin chromalum and photographed at the same magnification

- Fig 1* Third thoracic mammary gland from a newborn offspring of an alloxan diabetic rat. Distinct end buds are seen.  
*Fig 2* Third thoracic mammary gland from a newborn offspring of an alloxan diabetic rat. Distinct end buds. Distinct but small side buds.  
*Fig 3* Third thoracic mammary gland from a newborn offspring of a control rat. Distinct end buds are seen.  
*Fig 4* Third thoracic mammary gland from a newborn offspring of a control rat. Distinct end buds. Distinct but small side buds.


insulin and certain corticosteroids. The outcome of the present investigation suggests that the hormonal disturbance existing in offspring of alloxan diabetic rats is not reflected in the development of their mammary glands. The reason may be either that foetal mammary gland development is independent of at least some of those factors which regulate foetal growth of the body, or that factors promoting and factors inhibiting foetal mammary gland development are equally abundant in offspring of alloxan diabetic rats. It is interesting to note in this connexion that certain corticosteroids (11-oxysteroids), which seem to be present in excess in foetuses of non-insulinized alloxan diabetic rats, may exert an inhibitory action on the development of the mammary glands (Flux 1954).

## SUMMARY

The authors have studied the development of the mammary glands in newborn offspring of alloxan diabetic albino rats. No manifest differences from control offspring were noted. The observations are discussed in the light of our present knowledge regarding the hormonal imbalance in alloxan diabetic pregnant rats and their offspring.

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## NEOPLASMS IN GUINEA PIGS INDUCED BY AN AGENT IN ROUS CHICKEN SARCOMA<sup>1</sup>

By

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It has recently been reported that certain strains of Rous sarcoma virus are capable of producing sarcomas and haemorrhagic cysts in rats (2 to 7, 11, 13, 15-20), sarcomas in mice (2, 6) and hamsters (3), and fibrosarcoma like tumours and lymphogenous cysts in rabbits (2, 6, 19, 20). This paper describes the development of tumours in guinea pigs inoculated with Rous sarcoma material (Schmidt-Ruppini strain). A description will also be given of the growth of the guinea pig tumours *in vitro* as well as of the results of inoculation of guinea pig fibroblast cultures with Rous virus.

### MATERIAL AND METHODS

**Virus strains** Two strains of the Rous sarcoma virus were used. One strain, the Schmidt-Ruppini strain, was obtained from Dr H. Schmidt-Ruppini in Frankfurt A/M. It is capable of inducing neoplasia in rats, mice, hamsters and rabbits (2, 7, 11). The other strain, the Hill-Hall strain, was obtained from Sir Christopher Andrewes, National Institute of Medical Research, London. Rats, mice, hamsters and rabbits are not susceptible to this strain (4). Inoculation of either virus strain into chickens will within 7-10 days induce a neoplasm with the usual gross and microscopic appearance.

Guinea pigs were obtained by inoculation of rats for 1-2 weeks.

#### Animals

The guinea pigs (*Cavia Cobaya*) were purchased from a commercial dealer and inoculated intramuscularly in the right hind leg within the first week of birth (weight 70-240 grams). Adult guinea pigs weighing 375-700 grams were also inoculated. The guinea pigs were fed standard pellets and fresh greens. They were examined every third day for the first month after inoculation, then once a week or once a fortnight.

#### Inoculation

Two strains of virus were used. The virus was suspended in saline solution and inoculated into the guinea pigs.

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Ruppian strain) Of the 10 guinea pigs inoculated with repeated doses, all but one developed tumours. In only one of the adult guinea pigs did a tumour appear.

Of the 43 guinea pigs inoculated with cell free extract of the chicken sarcoma (Schmidt-Ruppian strain), 36 developed tumours. Positive results were obtained in all the 8 guinea pigs which had been inoculated repeatedly but negative in 2 of the 8 which had received the same total amount of extract in a single dose. 5 of the 8 guinea pigs that had received intraperitoneal injections of the extract developed tumours in the abdominal wall at the site of the needle puncture. No tumours were seen in the abdominal cavity.

When tumours developed, they invariably did so at the site of inoculation. They usually appeared in the form of a nodule or sometimes as a group of nodules 23-24 days after the inoculation but occasionally within 17 days. In some animals, however, the latent period covered up to 2-3 months.

In the initial stage the tumours increased only slowly in size. They were rounded and sometimes irregular, they felt firm and fibrous, and they were usually adherent to the surrounding muscles (Fig 1). The further development varied. After a period of slow growth the tumour often suddenly increased rapidly in size, expanded the thigh and bulged into the pelvis with consequent severe disability of the animal. At the same time the tumour became softer. In other animals the tumour became adherent to the overlying skin, which turned bluish-violet and finally ulcerated. Necrotic material was discharged from the floor of the ulcers (Fig 1). In some cases the tumour then regressed, in some the host died from a secondary infection. In others the tumour gradually became harder and smaller and left behind a fibrous scar, often with consequent permanent contracture of the hind leg. The size of the tumour ranged from  $4 \times 4 \times 4.5$  cm to  $0.5 \times 0.5 \times 0.5$  cm.

The cut surface was usually greyish-white with irregular areas of yellowish dry necrosis. Rapidly growing tumours had a moist white medullary cut surface and often showed central haemorrhages (Fig 2). The tumours were fairly well outlined against the surrounding tissue. In some cases metastases were seen in the lymph nodes adjacent to the tumours, in others the inguinal and retroperitoneal lymph nodes showed only an unspecific inflammatory reaction. The lungs in 4 guinea pigs were the site of numerous, hard, rounded, greyish-white tumour-nodules often surrounded by a narrow haemorrhagic zone (Fig 3). No metastases were seen in the liver, kidneys or spleen.

Histologically the tumour usually had the character of a spindle-cell sarcoma built up of densely crowded elongated cells, arranged in an irregular network of bundles (Fig 4). Often two types of cells could be distinguished (Fig 5). One type had a narrow nucleus fairly rich in chromatin, the cells were oblong and the cytoplasm scanty. The other type had a round, oval or slightly irregular nucleus with one or two

10 new born animals were inoculated on 6 successive days each day with 0.5 ml of sarcoma suspension. Sixteen adult guinea pigs were injected with 1 ml of sarcoma suspension 1.5.

Twelve guinea pigs were inoculated with a suspension of chicken sarcoma elicited by the Rous virus of the *Hill Hill strain*. Six of these were given 0.5 ml the other six 3 ml of a sarcoma suspension 1.5 in Hank's solution with antibiotics.

Forty-three new-born guinea pigs were inoculated with a presumed cell free extract of the Rous sarcoma (*Schmidt-Ruppin strain*). The chicken tumour was finely minced with a pair of scissors, then ground with glass powder for 15 minutes and suspended 1.5 in Hank's solution with antibiotics. The suspension was clarified in a VSI super mixer centrifuge for 15 minutes at 4000 r.p.m. The supernatant was transferred to an International cold centrifuge and centrifuged at 13800 r.p.m. (10000 g) for 30 minutes at  $-3^{\circ}\text{C}$ . The supernatant was cautiously removed and re-centrifuged for the same time and at the same rate. The procedure was repeated once more and the final supernatant obtained was used for inoculation. Of the 43 guinea pigs, 19 were given a single injection of 0.5 ml cell free extract, 8 a daily injection of 0.5 ml on 6 successive days, and 8 a single injection of 3 ml of extract. Light guinea pigs were inoculated intraperitoneally on 6 successive days each day with 0.5 ml extract.

Finally 6 new born guinea pigs were inoculated with 1 ml of chicken sarcoma extract (*Schmidt-Ruppin strain*) that had been passed through a Seitz filter.

### *Tissue Cultures*

Parts of the guinea pig tumours were finely cut with scissors and trypsinized in 0.1 per cent Trypsin Novo. The cells were cultivated in Carrel flasks containing Parker 199 with 2 per cent pooled inactivated calf serum to which was added 100 U of penicillin and 100 U of streptomycin per ml. The medium was changed every other day.

Fibroblasts from 30 day old guinea pig embryos were cultivated in the same way and inoculated with Rous virus (*Schmidt-Ruppin strain* and *Hill Hill strain*).

### *Occurrence of Haemagglutinins*

HA tests were carried out on blood cells from guinea pigs, chickens, sheep and human beings using supernatant obtained on centrifugation of homogenized guinea pig sarcoma suspended in saline or on supernatant from sarcoma cells cultured *in vitro*. No haemagglutinins were demonstrable. HI tests on sarcoma material and blood from guinea pigs harbouring tumours were kindly done by Dr H. O. Sjögren, Institute for Tumour Biology, Stockholm. The titre of haemagglutinin inhibiting antibodies did not indicate the presence of polyomavirus.

### *Serial Transplantation of the Guinea Pig Sarcoma*

Soft vital parts of the guinea pig tumour were minced with a pair of scissors and suspended 1.5 in Hank's solution with antibiotics. The coarser particles were allowed to settle. One millilitre of the turbid supernatant was injected intramuscularly into the right thigh of guinea pigs less than 2 weeks old.

### *Histological Technique*

Guinea pigs with tumours were sacrificed at various periods after inoculation. Pieces of the tumours, the inguinal retroperitoneal and mediastinal lymph nodes as well as parts of the liver, lungs and kidneys were fixed in Susa, embedded, sectioned and stained routinely with haematoxylin and eosin, sometimes with van Gieson, Masson's and Laidlaw's stain for reticulin.

## RESULTS

### *1 Injection of Material from Rous Sarcoma (Schmidt-Ruppin Strain)*

Tumours developed in 18 of the 23 new-born guinea pigs which had received a single injection of the Rous sarcoma suspension (*Schmidt-*

distinct nucleoli and sparse chromatin; the cells were round or of slightly irregular outline and the cytoplasm was most often relatively rich, pale or slightly eosinophilic. These two types of cells were often intermingled, but in the harder, fibrous tumours the former dominated, while the softer tumours were built up of free, rounded cells. In the latter case the tumour often had the character of an anaplastic round cell sarcoma (Fig. 6). More or less numerous connective tissue fibrils, readily recognized on silver impregnation were seen between the cells. Sometimes multinuclear giant cells were present, which gave the tumours a very polymorphous appearance (Fig. 7). Some tumours showed elongated band-like cells, whose cytoplasm was longitudinally striated (Fig. 8). The picture resembled that of a rhabdomyosarcoma, but no cross striation could be demonstrated.

The lymph nodes in the neighbourhood of the tumours were sometimes totally or partly invaded by tumour cells. No metastases were seen in the retroperitoneal or mediastinal lymph nodes. No cystic transformation of the lymph nodes was observed. The metastases in the lungs (Fig. 9-10 and 10) had the character of a spindle-cell sarcoma and were built up of elongated cells with oval nuclei. In many metastases the cells surrounded the bronchi like a cuff, but sharply demarcated collections of sarcoma cells were also seen in the lung parenchyma. Central areas of necrosis and inflammatory infiltrates were seen in the larger tumour nodules in the lungs. No secondary growths could be detected in the liver, spleen or kidneys.

All attempts to induce sarcomas in new born guinea pigs with Seitz filtrates of extracts of the chicken sarcoma (Schmidt-Ruppin strain) failed.

## 2 Serial Transfer of the Guinea Pig Sarcoma

Six 1-2 week old guinea pigs were inoculated intramuscularly with 0.5-1 ml of a 1-0 week old guinea pig tumour suspended 1:5 in Hank's solution with antibiotics. Within 11 days 4 of the injected animals developed a  $2 \times 2 \times 2$  cm tumour at the site of infection. These tumours rapidly increased in size. The tumours were soft and their cut surface was greyish-white and moist. No metastases were seen. Pieces of one of the tumours were finely minced and suspended 1:5 in Hank's solution. One millilitre was injected intramuscularly into the right hind leg of 5 guinea pigs weighing about 250 grams. Takes were obtained in

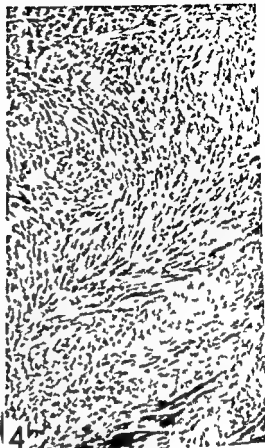
Fig. 1 Ulcerated tumour in the hind leg 6 weeks after -

Fig. 2 " " " " " " " " " " " "

Fig. 3 " " " " " " " " " " " "

Fig. 4 " " " " " " " " " " " "





Figs 1-5



Figs 9-10

Fig 9 Metastases in the lungs of a guinea pig inoculated intramuscularly in the right hind leg with material from fowl chicken sarcoma  $\times 13$

Fig 10 The metastases in the lungs are built up of closely packed spindle cells Hix eosin  $\times 130$

4 of them, in which the tumours attained the size of  $3 \times 3 \times 2$  cm within 10 days (Fig 11). Material from one of the tumours was transferred to 4 guinea pigs weighing 300-400 grammes. Takes were obtained in 2 animals, but the growth of the tumour was slower than before. On further passage to 4 new guinea pigs only temporary growth was obtained and the tumours regressed after a week.

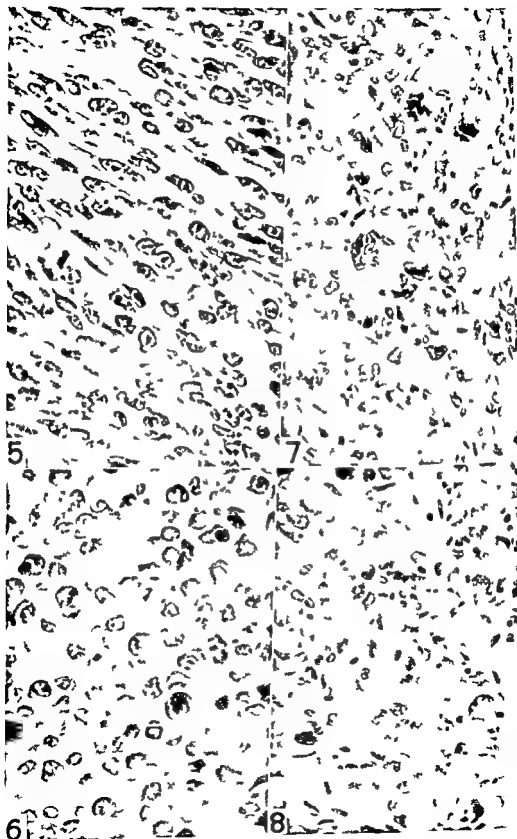
During the first passages all guinea pigs inoculated with the tumour died. The liver often showed more or less extensive necroses and one of the animals had focal necroses in the spleen. Treatment of the animals with chloromycetin did not ward off the fatal issue.

Fig 5 Two types of cells can often be distinguished in the guinea pig sarcoma: one with a narrow nucleus fairly rich in chromatin, another with a round or oval nucleus with sparse chromatin and one or two distinct nucleoli. Hix eosin  $\times 450$ .

Fig 6 Guinea pig sarcoma with the appearance of a round cells sarcoma. Hix eosin  $\times 450$ .

Fig 7 Guinea pig sarcoma of pleomorphic character. Hix eosin  $\times 350$ .

Fig 8 Guinea pig sarcoma with numerous elongated cells whose cytoplasm was longitudinally striated. Hix eosin  $\times 400$ .





Figs 9 10

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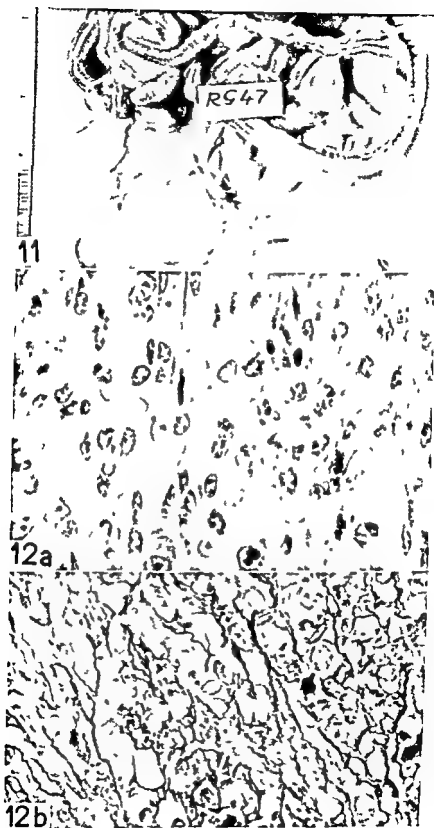
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Fig 5 Two types of cells can often be distinguished in the guinea pig sarcoma one with a narrow nucleus fairly rich in chromatin another with a round or oval nucleus with sparse chromatin and one or two distinct nucleoli Htx eosin  $\times 480$

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Fig 7 Guinea pig sarcoma of pleomorphic character Htx eosin  $\times 350$

Fig 8 Guinea pig sarcoma with numerous elongated cells whose cytoplasm was longitudinally striated Htx eosin  $\times 400$



Figs 11-12

Histologically the tumour in the first guinea pig was an anaplastic medullary sarcoma built up of slightly irregular cells with oval or round nuclei and a sparse cytoplasm (Fig 12). On silver staining a network of argentophilic fibrils was seen between the cells. Numerous mitoses were observed. The structure was maintained during the successive passages apart from an admixture of leukocytes in some of the tumours. The number of mitoses in the material used for the last passage was still rather high.

Several attempts to transplant guinea pig tumours of spindle cell sarcoma type have so far failed.

All attempts to transfer the guinea pig sarcoma to guinea pigs by means of supernatant fluid from centrifuged guinea pig tumour homogenates suspended in Hank's solution have proved unsuccessful.

### 3 *Back Transfer of the Guinea Pig Sarcoma to the Chickens*

In a series of guinea pigs 0.5 ml chicken sarcoma suspension was injected into the right thigh. One week later the injected thigh muscles of one of the animals were excised and saline extract from the excised muscles was injected into the breast muscles of 4 chickens. All of them developed typical Rous sarcoma at the site of injection and metastases in the lungs, liver and heart. Another guinea pig was killed one month after the inoculation at which time it had a  $2.5 \times 2.5 \times 2$  cm tumour in the thigh. 0.5 ml of a finely minced suspension of the tumour was injected bilaterally into the breast muscles of 3 chickens. One of these developed a sarcoma of Rous type on one side of pectus carinatum. No tumour appeared on the other side or in any of the other inoculated chickens. All attempts to transfer the tumour to chickens with the use of material from guinea pig tumours older than one month calculated from the date of inoculation of the animal with chicken tumour material have failed. Nor have repeated attempts to transfer the passage guinea pig tumour to chickens met with any success.

### 4 *Injection of Material from Rous Sarcoma (Strain Mill Hill)*

The Rous sarcoma of Mill Hill strain was passed at 2 week intervals through a series of 3 week old chickens. A saline suspension of the tumour prepared in the same way as the material from Rous sarcoma of Schmidt Rupp strain was given to 12 newborn guinea pigs (150-160 grammes). No tumour appeared in any of the inoculated animals. They were observed for 4 months.

Fig 11 See 2nd passage of a transplanted guinea pig sarcoma.

Fig 12 The transplanted guinea pig sarcoma had a rather anaplastic character. Sparse argentophilic fibrils were seen between the sarcoma cells. A. H. T. x 480.

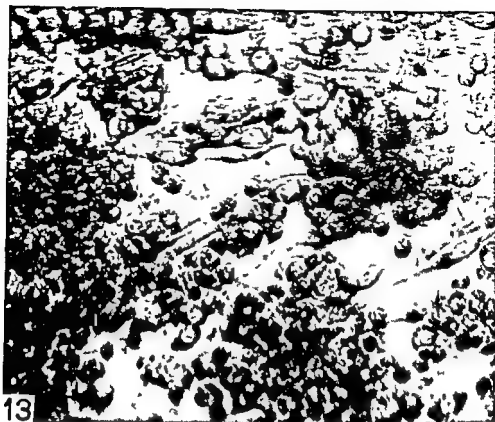


Fig 13

Multilayered foci of rounded cells with granulated cytoplasm appearing in cultures of embryonal guinea pig fibroblasts inoculated with Rous chicken sarcoma virus (Schmidt Ruppian strain)

### 5 Tissue Culture Experiments

Cells isolated from the guinea pig tumours and dispersed in trypsin grew during the first week as a monolayer. Then multilayered foci of rounded cells with granulated cytoplasm appeared (Fig 13). Similar plaques appeared after 9 days in cultures of embryonal guinea pig fibroblasts inoculated with Rous virus (Schmidt-Ruppian strain).

No plaques appeared in cultures of embryonal guinea pig fibroblasts inoculated with the Mill Hill strain of Rous virus.

### DISCUSSION

Various investigators have tried to transplant Rous chicken sarcoma to the anterior chamber of the eye (14) or to the brain (1, 9) of guinea pigs. Only temporary growth was obtained. No growth was seen in our experiments in which cellular suspension of Rous sarcoma of Mill Hill strain was transferred to new born guinea pigs.

In contradistinction hereto tumours developed in about 75 per cent of the guinea pigs inoculated with material from Rous sarcoma of Schmidt-Ruppian strain. For several reasons we think that these tu-

tumours are not the result of proliferation of the implanted cells but induced *de novo* by the effect of some agent in the tumour material. Heterotransplantation of cancer cells is difficult unless the innate resistance of the recipients is modified by treatment with x-rays, cortisone or agents having similar effects. No such treatment was given to our animals. Tumours were obtained not only after transfer of cellular suspension of the chicken tumour, but also after injection of a presumably cell-free extract of the tumour. Preliminary chromosome analysis of the guinea pig sarcoma cells by *Professor Albert Levan* showed chromosomes of guinea pig type. No cells with chromosomes of bird-type were observed. The oncogenic effect on rats, mice, hamsters and rabbits of Rous sarcoma virus (Schmidt-Ruppin strain), has been demonstrated in previous investigations (2-7, 11). In rats no serological evidence of any antigen common to the rat sarcoma and the chicken sarcoma could be found (5, 10). The chromosomes of the sarcomas in rats and mice had the character of rat and mouse chromosomes, respectively (5, 11, 12). It was concluded that the tumour in rats, mice and hamsters were built up of the hosts' own cells and that the tumours had been induced by a virus. There is no reason to assume that the oncogenic mechanism in the guinea pigs should differ from that in the other mammals.

The nature of the test carried out was a variant (mutant) test carried out from Rous sarcoma of Mill Hill strain and of Schmidt-Ruppin strain,

The presence of polyoma virus have been found in the Rous tumour carrying mammals or in chickens inoculated with the Rous virus. The latency, i.e. the time between the inoculation of the virus and the first palpable tumour, is much shorter in Rous virus infected guinea pigs than after infection with polyoma virus. Inoculation with polyoma virus will induce tumours also in the liver, adrenals, kidneys and spleen, i.e. in organs never found to harbour neoplasms in our guinea pigs. Apparently unlike polyoma virus induced neoplasms Rous sarcomas in guinea pigs sometimes regress spontaneously. The structural resemblance between the two types of virus tumours in guinea pigs might be due to the connective tissue reacting in the same way to different oncogenous viruses.

The guinea pig tumours and the tumours obtained in the other mammals inoculated with Rous virus (Schmidt-Ruppin strain) are similar



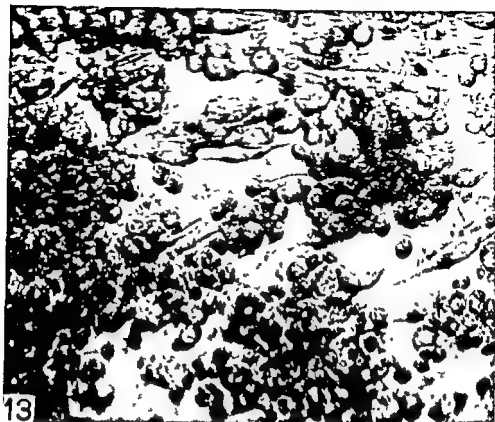


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The nature of the causal agent is still unknown but it is most likely a variant (mutant) of the classical Rous sarcoma virus. Neutralization tests carried out with anti serum from rabbits immunized by material from Rous sarcoma of Mill Hill strain and of Schmidt-Ruppin strain, respectively, indicate that the virus strains are closely related but not identical (2). However, the possibility of contamination of the chicken sarcoma with some other virus such as SE polyoma virus must also be considered, especially as the spectrum of susceptibility in mammals seems to be the same for both viruses. In guinea pigs the polyomavirus induces, at the site of inoculation, undifferentiated sarcomas, composed mainly of spindle shaped cells (8). However, no HA or HI antibodies indicating the presence of polyoma virus have been found in the Rous tumour carrying mammals or in chickens inoculated with the Rous virus. The latency, i.e. the time between the inoculation of the virus and the first palpable tumour, is much shorter in Rous virus infected guinea pigs than after infection with polyoma virus. Inoculation with polyoma virus will induce tumours also in the liver, adrenals, kidneys and spleen i.e. in organs never found to harbour neoplasms in our guinea pigs. Apparently unlike polyoma virus induced neoplasms Rous sarcomas in guinea pigs sometimes regress spontaneously. The structural resemblance between the two types of virus tumours in guinea pigs might be due to the connective tissue reacting in the same way to different oncogenous viruses.

The guinea pig tumours and the tumours obtained in the other mammals inoculated with Rous virus (Schmidt Ruppin strain) are similar

in many respects. They all appear at the site of injection and after a relatively short period of latency. Histologically they often have the character of spindle cell sarcoma. Two types of cells, one spindle shaped the other rounded or irregular "macrophage"-like has been distinguished in rat, mice, hamster and guinea pig tumours. This is noteworthy because two types of cells principally similar to those in the mammalian tumours can also be distinguished in Rous sarcoma in chickens.

On the other hand, in guinea pigs the reaction to the Rous virus was not identical with that in rats, mice and hamsters. Common findings in rats were cysts, usually situated in the groin, axillae and neck and most often filled with haemorrhagic fluid (2, 3, 5, 15-18). Similar lymphogenic cysts have been seen in rabbits (19, 20) and occasionally in hamsters (personal observation). No cysts were seen in the guinea pigs. The sarcoma is often metastasized in the rats, mice and hamsters, but rarely in the guinea pigs. In the former animals the tumours always grew progressively, in guinea pigs they sometimes regressed. In the rabbits the tumour always disappeared within some weeks. It thus appears that the reaction to the Rous virus is fundamentally the same in the different mammals but differs in some respects from species to species.

The sarcomas could be readily carried in series through rats, mice, and hamsters respectively (1-6, 15-18). The tumour could also sometimes be successfully transplanted in series in guinea pigs, but not through more than three passages. It is possible that the guinea pig tumour has less growth energy than the tumours in the other mammals. The rats, mice, and hamsters all belonged to a closed colony kept at the institute for a long time, whereas the guinea pigs were purchased from various dealers. They therefore possibly had a more heterogeneous character than the other animals and this might also influence the growth of the transplanted tumour.

On back transfer of the rat, mice, and hamster sarcoma to chicken a tumour developed at the site of injection, which had the usual appearance of a Rous sarcoma (1-6, 15-16). The same result was obtained after injection of material from Rous tumours passed in series in the above-mentioned mammals. Thus, in our laboratory the Rous virus has been recovered from the 72nd passage tumour in rats, the 47th passage tumour in mice and the 7th passage tumour in hamsters. Svoboda (18) has recovered his Rous virus from rat sarcomas passed through 45 generations. But all attempts to recover the virus from Rous guinea pig tumours carried in series in guinea pigs failed. In guinea pigs injected with Rous virus the virus was found at the site of injection one week after the inoculation. Its amount or activity was substantially reduced one month after the inoculation (in a 12 day old guinea pig tumour). In spite of numerous attempts the virus could not be demonstrated in older guinea pig tumours.

Not even injection into chickens of material from guinea pig tumours which had been grown in tissue culture for several weeks and in which the increase of the virus had not been hampered by antibodies was followed by a tumour

The failure to recover the virus from the guinea pig tumours raises many questions. One might imagine that the virus is present in a latent or inactive form or in too small an amount to elicit tumours in a new animal. It is also possible that the virus only initiated the guinea pig tumours and that the further growth of the tumours occurs independently of the virus. Answers to these and related questions must abide future research.

### SUMMARY

New born guinea pigs were inoculated intramuscularly with a suspension of finely minced Rous chicken sarcoma (Schmidt Rupp strain) or with presumed cell free supernatant fluid obtained by repeated centrifugation of suspension of homogenized chicken sarcoma. In about 75 per cent of the guinea pigs tumours developed at the site of injection within 3 to 4 weeks. They usually grew progressively. In a few cases secondary growths appeared in the lungs. Sometimes the tumours spontaneously regressed. Histologically they usually had the character of spindle cell sarcomas, sometimes of round cell sarcomas or pleomorphic sarcomas.

The guinea pig sarcoma was successfully transplanted to new born guinea pigs and could be passed in series through 3 generations. All attempts to transfer the sarcoma from guinea pig to guinea pig by means of cell free material from the guinea pig sarcoma failed. Material from 14 day old guinea pig sarcoma (1 month after the inoculation of the virus) injected into chickens elicited a Rous sarcoma at the site of the injection. All attempts to recover the virus from older guinea pig tumours failed.

Embryonal guinea pig fibroblast inoculated *in vitro* with Rous sarcomavirus (strain Schmidt Rupp) developed plaques within 9-10 days. No such plaques appeared after inoculation with Rous sarcoma of Mill Hill strain.

No tumour appeared in guinea pigs inoculated with Rous sarcoma of Mill Hill strain.

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## VIRAL SUSCEPTIBILITY AND EMBRYONIC DIFFERENTIATION

### 1 *The Histopathology of Mouse Kidney Rudiments Infected with Polyoma and Vesicular Stomatitis Viruses in vitro*

By

LAURI SÄTÉN, TAPANI VAINIO and SULO TOIVONEN

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The word "competence" has long been used by embryologists to indicate the capacity of cells and tissues to respond to environmental conditions which lead to changes in the expression of their genetic potentialities. During normal development, this response may be termed determination. Under experimental conditions we are as a rule dealing with a change in the prospective determination. Because of the lack of specific markers the epigenetic factors studied to date have mainly been factors and conditions which lead to a change in the morphology of the cells and thus belong to the category of "differentiating" factors. During foetal life the capacity of the cells to respond to such morphogenetic stimuli undergoes restriction, although in a broader sense most of the cells in an adult organism continue to exhibit some competence. The metaplasia of differentiated cells can be taken as one such example, and perhaps the malignant transformation of cells is indicative of a certain competence to respond to environmental factors.

As was mentioned above, our present knowledge of the changes in cellular competence is principally based upon studies on

... and factors accordingly we have initiated a series of experiments with another epigenetic factor, the virus, and have analysed the changes in "viral competence" during embryonic differentiation. A large amount of clinical data and the results of experiments made by teratologists, have suggested that such changes in viral susceptibility do occur during foetal life (reviewed Ebert & Wilt

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The authors wish to thank Mrs. Anja Tuomi, Miss Ann Kristin Thors and Miss Lissa Helleman for technical assistance.

1960, Tondury, 1962) These changes have been correlated to certain stages in normal development, but their background and mechanism are unknown Consequently, we have endeavoured to find new examples of such changes, and especially in organs where the development and its normal control mechanisms are well known Earlier reports of the effect of polyoma virus upon certain tissues (Dawe 1960 Dawe *et al* 1962) together with the investigations of Grobstein (1953 1956 1961) on the development of the kidney rudiment *in vitro* seemed to provide an opportunity for the initiation of such studies

## MATERIAL AND METHODS

*Mice* Random bred Swiss mice were used Eleven to 16 day embryos were used and the 0 day was evidenced by vaginal plugging

*Organ cultures* The kidneys of the embryos were removed and placed on a Trowell type metal screen on a Millipore filter (for detailed description see Saxe *et al* 1962) In some experiments the rudiments were cultivated direct on a collagenized cover slip (Lainio 1959) Eagles basal medium in Earles balanced salt solution was used with 10 per cent calf serum The medium contained 0.0172 M of sodium bicarbonate and the cultures were incubated in 5 per cent  $\text{CO}_2$  in air

*Viruses* The polyoma virus utilized in these studies was prepared in mouse fibroblast cultures The HA titre of the stock virus was 1:1024, when newborn litters of random bred Swiss mice were infected with this virus preparation it caused a tumour frequency of 80 per cent over an observation period of 6 months

Vesicular stomatitis virus (VSV) was obtained through the courtesy of Dr Karl Cantell of the State Serum Institute Helsinki It was prepared in a human amnion cell line (U cells) The stock virus had a titre of  $10^{8.3}$  pfu/cc when titrated in chick embryo fibroblasts The same stock of VSV stored at  $-40^\circ$  was used throughout the experiments

*Infection of the cultures* The cultures were adsorbed for one hour with either of the viruses 5 cc of viral dilution was used and not more than 20 organ rudiments were placed in this volume The polyoma virus was used in 10:1 dilution from the original stock virus the corresponding dilution of VSV being 10:1 After adsorption the cultures were thoroughly washed in phosphate buffered saline to which had been added 10 per cent calf serum and thereafter transferred to culture dishes

*Microscopy* For fixation the rudiments were carefully removed from the filter paper (or the cover slip) and kept for 30 min in neutral 10 per cent formalin The specimens were dehydrated in butanol series embedded in paraffin and serially sectioned Different staining methods were used one of the most suitable seemed to be Mayers haemalum plus periodic acid Schiff stain

*Photography* In addition to the usual microphotograms the cultures were photographed frequently under living conditions The collagenized cover slips provided excellent optical conditions for this purpose

*Experimental series* The histopathology of the lesions caused by polyoma infection was followed in 369 kidney rudiments The detailed description of these series has been published earlier (Saxe *et al* 1962)

As regards the series infected with VSV the kidneys mainly used were from 14 day embryos After infection the kidneys were fixed after 18 20 24 32 36 40 and 48 hours three kidneys being investigated on each occasion The series was repeated twice In addition 33 rudiments were used for special stainings after 24 hours cultivation

## RESULTS

*Normal development* The main features of the development of mouse kidney rudiment under our experimental conditions need a brief description When removed from a 12-day embryo, the metanephro

genic mesenchyme is morphologically undifferentiated, although the typical shape of the metanephros can be seen in the mesenchymal condensation. The ureteric bud has already reached the metanephrogenic mesenchyme, and has in most cases formed the first branches. Following subsequent cultivation for 24 hours, the ureteric bud has branched twice or three times, and the onset of tubulogenesis is seen as a condensation of the peripheral metanephrogenic mesenchyme (Fig. 1). After a total period of cultivation of 48 hours, the first tubuli are seen in gross examination. They are located between the peripheral, undifferentiated mesenchyme and the central loose mesenchymal tissue, and are visible as small applelike formations (Fig. 2). During subsequent cultivation, new tubuli are continuously being formed between these two areas, and simultaneously a more delayed tubulogenesis is seen in the peripheral parts (Figs. 3 and 4). Thus, between 2 and 12-14 days a series of tubules in different developmental stages is seen in all the cultures: the youngest condensations are the most peripheral, and the oldest tubuli with definite cytodifferentiation are located in the central portions (Figs. 1 to 4). After two weeks of cultivation, the formation of new tubules seems to cease, and the tubuli attain their typical S shape and become connected with the collecting system.

Without going into details of the histogenesis, it is important to describe the different tissue components seen in all of these rudiments. This is illustrated in Fig. 5. The distinction between these tissue components must be effected before a study is made of the more or less specific distribution of viral lesion in the kidney rudiments.

*The histopathology of the polyoma lesions.* No more than a summarized description of the effect of polyoma virus on the kidney rudiment is presented below. The observations have been presented in detail in a previous paper (Saxen et al. 1962).

The various tissue components mentioned above show definite differences in their viral susceptibility. The loose central mesenchyme is affected first, resulting in the appearance of degenerating cells and cell debris between the branches of the ureteric bud and the developing secretory tubules (Figs. 6 and 7). The condensed areas, along with the kidney tubules at different stages of development, seem to resist the pathogenic effect of the virus for a rather long period. When the first degenerative changes in the undifferentiated mesenchyme become discernible two days after infection, the epithelial cells are still morphologically normal after a subsequent cultivation of 10-12 days.

It is difficult to judge whether we are dealing with a direct effect of the virus, or whether the changes can be attributed to the unsuitable environmental conditions provided by the lytic tissue.

As concerns the cytology of the viral lesions caused by polyoma virus, not much can be added to our very scanty information on viral cyto-





Figs 1-4

Four developmental stages of mouse kidney rudiment cultivated *in vitro*, and photographed in living conditions on a collagenized cover slip. Rudiments were removed from 12 day embryos and cultivated for one (Fig 1), three (Fig 2), seven (Fig 3), and 10 days (Fig 4) respectively. The onset of tubulogenesis is seen as condensation of peripheral metanephrogenic mesenchyme and the secretory tubuli appear as sharply demarcated balls in the more central parts of the rudiments in Figs 2-4.

**pathology.** In addition to nuclear pycnosis leading to complete disintegration of the nuclei and to the appearance of Feulgen-positive debris, nuclei "ballooning" and inclusion bodies are seen in the mesenchymal cells. Furthermore, paranuclear vacuolization is noted in some cells, especially in the epithelial cells of the old, disintegrating cultures.

**The histopathology of the VSV lesions.** The present series of microphotographs gives us an idea of the main features of the lesions caused by VSV under our experimental conditions. Against what was said above relating to the CPE of the polyoma virus, the higher pathogenicity of the VSV is obvious. First lesions were obtained after 18-20 hours, and in all the series the kidneys were completely disintegrated after 48 hours of cultivation. These observations were made in 14-day

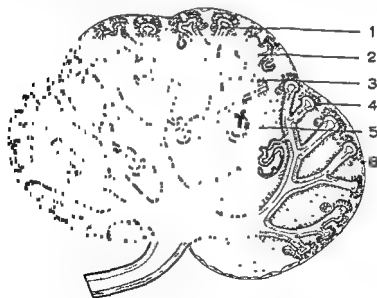
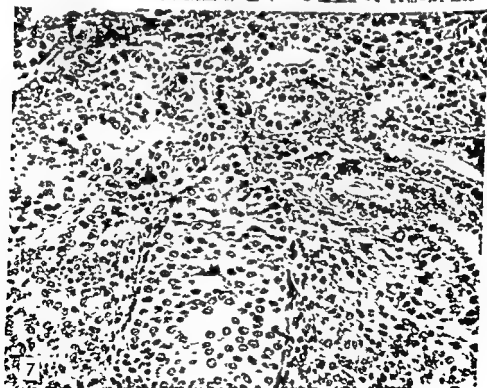
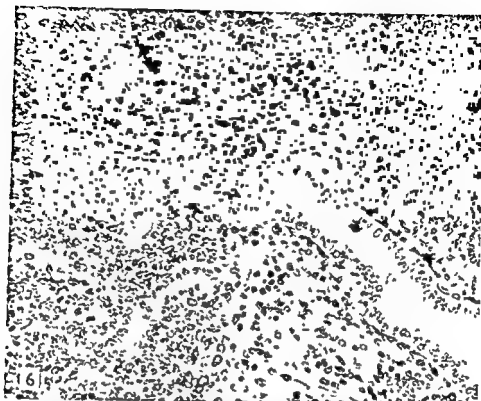


Fig 5

mesenchyme

(or) capsular



kidneys, when younger rudiments from 12 day embryos were used, the destruction was even more rapid

As was the case in polyoma-infected kidneys, definite differences in the susceptibility of different tissue components became evident—although in a somewhat different way. The first lesions were now observed in the *condensed mesenchyme* around the ends of the collecting tubes (Figs 9, 10, 14, 15). This was observable in both the peripheral condensations and the older, central pre-tubular condensations which did not show epithelial differentiation. In contrast, the tubular structures and the branches of the collecting system seemed to resist the viral effect at this stage of infection (18 to 30 hours subsequent to infection). At this time the loose mesenchyme between the condensed areas gradually became affected, whereas the mesodermal sheath around the collecting tubes was morphologically intact. Subsequently, the secretory tubules also showed the first degenerative changes, but here again, not before they had been surrounded by dead or dying mesenchymal cells. At the final stage, even the well-differentiated epithelium of the collecting system exhibited degeneration.

The cytological changes consist in the main of nuclear pyknosis and disintegration. However, when a comparison is made with our observations on the effects of polyoma virus, certain differences are noticeable. Nuclear ballooning, described by us, and many investigators before us (Stewart 1960, Dawe & Law 1959, Stanton *et al* 1959), in polyoma-infected cultures is lacking or at least very rare in cultures infected with VSV. In contrast, the "perinuclear vacuolation" described by Weiss & Kirsten (1962) in polyoma infection was quite exceptional in our polyoma cultures, but common in the kidney cells infected with VSV. In the secretory tubules surrounded by cell debris in particular, this type of lesion appeared to be the most common (Fig. 16).

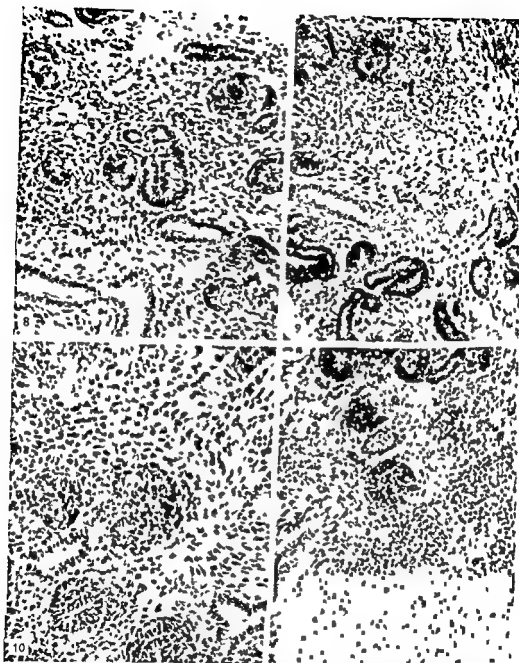
By way of conclusion, it may be added that similar or other degenerative changes were rare in the control series cultivated under identical conditions without infection. On occasion, some dying mesenchymal cells could be seen in the serial sections of the younger cultures. In cultures kept *in vitro* for more than 12 days, the central parts of rudiments showed more advanced lesions, obviously due to unfavourable nutritional conditions. However, these lesions were clearly distinguishable from the viral cytopathic effects.

Fig 6 and 7

Two kidney rudiments —  
lesions consist

The  
un-  
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cul

\* rudiment cultivated  
and periodic acid—



Figs 8-13

A series of microphotographs showing the viral lesions in a 14 day mouse kidney rudiment infected with vesicular stomatitis virus (VSV) and subsequently cultivated for 20 24 28 32 36 and 40 hours. The first lesions are seen in the peripheral condensations and subsequently in the stromal mesenchyme. Not until 10 hours later are the secretory tubuli affected and even after 36 to 40 hours cultivation subsequent to the infection the collecting tubes and their mesenchymal sheath seem morphologically normal. Haematoxylin and periodic acid—Schiff  $\times 200$ .

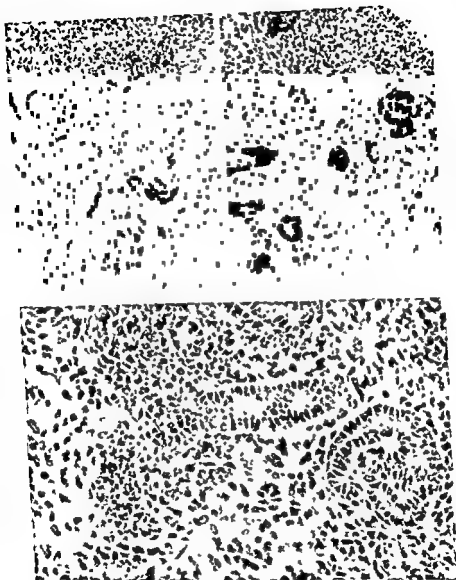
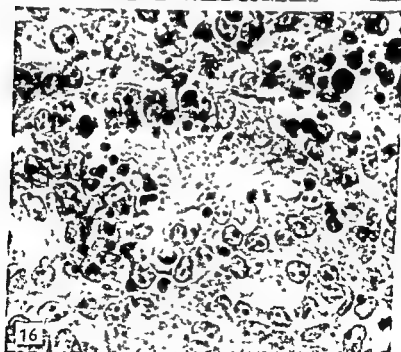
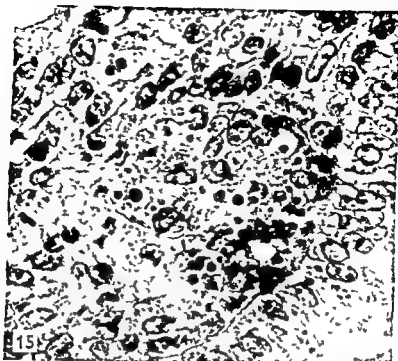


Fig 14

A detail of a 14 day kidney rudiment infected with VSV and subsequently cultivated for 22 hours. From right to left the figure shows the main components in the nephrogenic tissue: a secretory tubulus, a collecting tube with its two branches, metanephric condensation around the tips of these branches, and the loose stromal mesenchyme in between these components. The viral lesions are seen in the mesenchymal condensations. Haematoxylin and eosin.  $\times 350$



*Figs 15 and 16*

- Fig 15* A typical early lesion by VSV in a mesenchymal condensation close to the collecting tube. Pycnotic nuclei and nuclear debris (pseudoinclusions) are to be noted. Haematoxylin and periodic acid—Schiff  $\times 700$
- Fig 16* Late viral lesions in a kidney rudiment infected with VSV. The secretory tubulus is surrounded by cellular debris and the epithelial cells of the tubulus show degenerating nuclei and paranuclear vesiculation. Haematoxylin and periodic acid—Schiff  $\times 700$

## DISCUSSION

The results presented above show that there are definite differences in the viral susceptibility, the "viral competence" of the embryonic cells in one and the same organ rudiment. Furthermore, they show that changes in this susceptibility do occur during development. Metanephrogenic cells are highly susceptible before the onset of cytodifferentiation, but resist the viral CPL after getting involved in the tubulogenesis. In addition, the results indicate that there are already agent-specific differences present in the viral susceptibility at an early stage of development: the condensed, pretubular mesenchyme seems to resist the CPL of polyoma virus, but is highly susceptible to the pathogenic effect of vesicular stomatitis virus. It is unnecessary to stress that this study constitutes but a very limited example of viral effects on embryonic tissues, and represents no more than the start of a more extensive series of investigations along these lines. Moreover, this part remains purely descriptive in nature, and follows the classical lines of histopathology. Nevertheless, some questions can be brought up in this connection and we should like especially to compare our results with the present knowledge of experimental teratology.

In recent years, experimental teratology has yielded abundant evidence for the classical concept of *Stockard* (1921) which states that foetal lesions due to environmental factors are largely dependent upon the developmental stage of the pathogenic treatment: different teratogenic agents result in similar anomalies when applied at the same stage of development, whereas the same factor leads to different types of lesions at different stages of foetal development.

The importance of the stage of development of teratogenic treatment has been demonstrated in many investigations concerning foetal lesions brought about by viral infections. The voluminous literature on the effect of maternal rubella on the human foetus indicates that the child can be damaged only when the disease is contracted during the classical sensitive period, the first trimester of pregnancy. Furthermore, the localization of the viral lesion (lens primordium, ear vesicle, heart, etc.) seems to depend on the time of the rubella infection (reviewed by Swan 1946, *Mayes* 1957, *Siegel & Greenberg* 1960, *Tondury* 1962). Experimental results with different viruses have confirmed these ideas.

In a number of experiments with influenza A virus stress has been laid on the importance of the time of infection (*Hamburger & Habel* 1957, *Williamson et al.* 1953). The observations of *Kana* (1942) and *Burnet* (1950)

former

ceptible

... virus, whereas the latter demonstrated that some 10 days later this susceptibility was restricted to the lung primordium. Corresponding observations have been reported from experiments with Newcastle disease virus (*Blattner & Williamson* 1951, *Williamson et al.* 1953) and with vaccinia virus (*Thalhammer* 1957).



When the different factors responsible for such "sensitive periods" for viral damage are under discussion, a great number of conditions have to be considered. The penetration of the virus into the foetal tissues is naturally of great importance, and it has been suggested that tissues are affected when they are exposed to the exterior at the time of inoculation (Saxen 1958, Ebert 1961, Hamburger 1961). The state of the target tissue has also been mentioned as an important factor. As in discussion of the sensitive periods of other teratogenic factors, the metabolic state and the proliferation rate of the infected tissue have been put forward as having some bearing on the viral susceptibility (Williamson *et al* 1953, Robertson *et al* 1955). However, as Ebert & Will (1960) have pointed out, all direct evidence in favour of this "general feeling" is lacking as far as viruses are concerned.

In referring to the complicated problem of viral embryopathies, Ebert (1961) recently stated, "There is a compelling need for systematic studies and comparisons of the effects of inoculations of specific viruses into the embryo at successive intervals during ontogeny, under uniform conditions of dosage and other controllable conditions". We do not think that our experimental conditions can meet all the prerequisites he states as necessary, but nevertheless it seems that such *in vitro* studies on organ rudiments can give us a valuable tool for certain comparative investigations under controlled conditions. Of course, this does not imply that results obtained *in vitro* are applicable as such to *in vivo* conditions, as was pointed out in our earlier report (Vainio *et al* 1963).

In pursuance of the old statement by Stockard (1921), it can be concluded that the idea of certain sensitive periods is valid even at a cellular level, as is evidenced by the changes in the susceptibility of the mesenchymal cells in the metanephrogenic anlage. However, our results indicate that the quality of the teratogenic agent cannot be neglected: there were marked definite differences in the cellular specificity of the effect of the two viruses so far studied. The causes of these differences are hardly attributable to the experimental conditions, which were identical in the different series, and thus explanation of the time-dependent and agent-specific susceptibility must be sought in the target tissue itself. Such factors as the presence or the absence of virus receptors, the capacity to support viral replication, the rôle of the metabolic activity and the proliferation rate, etc., need investigation. Our experimental conditions seem to afford an opportunity to do so, and some results of investigations along these lines will be presented in the next papers of this series.

#### SUMMARY

The paper deals with the histological changes noted in mouse kidney rudiment *in vitro* after infection with SE polyoma virus and vesicular stomatitis virus (VSV). The microscopical lesions are described at the different intervals of the *in vitro* cultivation of infected rudiments.



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## VIRAL SUSCEPTIBILITY AND EMBRYONIC DIFFERENTIATION

### 2 Immunofluorescence Studies of Viral Infection in the Developing Mouse Kidney *in vitro*

TAPANI VAINIO LAURI SAXÉN and SULO TOIVONEN

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It was inferred from the experiments presented in our previous paper that vesicular stomatitis virus (VSV) attacks the developing mouse kidney *in vitro* and damages the mesenchymal condensation areas in particular (Saxén *et al* 1963). This is sharply opposed to the CPE by polyoma virus in the same organ *in vitro* (Saxén *et al* 1962). The condensation areas seem to be resistant to the CPE of polyoma virus. However, it must be emphasized that VSV is a toxic virus and may destroy the tissues in which it does not replicate (Cantell *et al* 1962). Thus the CPE found in the mesenchymal condensations in VSV infection does not necessarily indicate that the virus replicates there which does in fact determine the susceptibility to a given virus.

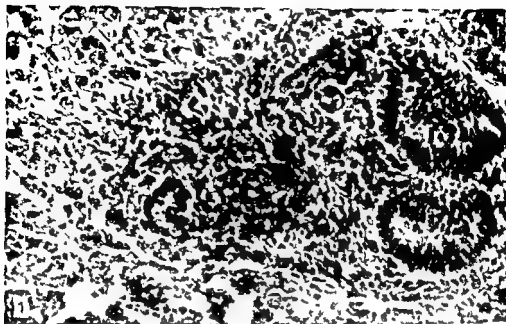
To gain some knowledge of which tissue components in the developing mouse kidney support the replication of VSV, some investigations were made with the aid of fluorescent antibodies which had been successfully used in studies with polyoma virus (Vainio *et al* 1963). In conjunction with this, some comparisons were made between the foetal kidneys infected with VSV or polyoma virus *in vitro* with the distribution of the viral antigens being taken into consideration.

## METHODS

- Organ cultures* were prepared in the way previously described (Saxén *et al* 1962) with the culture medium given in the previous paper.
- Viruses* The VSV and polyoma virus utilized in these studies were from the same stock as those described in the previous paper.
- Infection of cultures* Organ rudiments were adsorbed with VSV and polyoma virus using the dilutions and procedures given earlier.
- Immunofluorescence* Antisera against polyoma virus were prepared in rabbits.

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The authors wish to thank Mrs Anja Tuomi, Miss Ann Kristina Thors and Miss Liisa Heilemaa for technical assistance.



*Figs 1 and 2*

The section and the corresponding fluorogram of a fetal mouse kidney infected with polyoma virus and cultivated for 8 days *in vitro*. Note the stained nuclei in the cells between the tubuli and the absence of fluorescent material from the tubuli cells themselves.

by weekly injections of the stock virus (Lainio *et al* 1963). The intraperitoneal route a month of immunization the virus was given ant. The conjugation to fluorescein isothiocyanate (FITC) was performed according to Marshall *et al* (1958). Rabbit acetone powder

was acquired through the courtesy of Dr Kurt Paucker, the Children's Hospital of Philadelphia, USA. It had been prepared



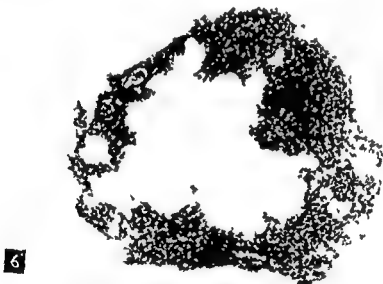
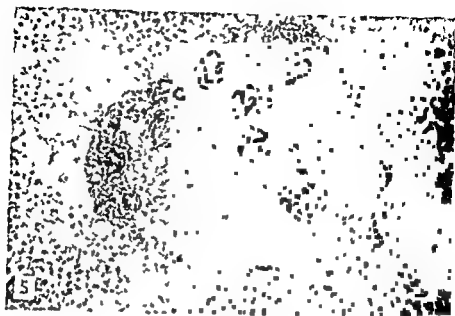
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**FIGS 3 and 4**

The section and its fluorogram of a 14 day old foetal mouse kidney infected with VV and subsequently cultivated for 22 hours. The periphery of the organ shows an abundance of the antigen, some tubuli remain unstained as do the mesenchymal cells in the middle of the organ.

absorptivity with rabbit acetone powder was done shortly before the conjugate was to be used.

1. The first of these is the fact that the Commission has not yet received any information from the Government of the United States regarding the results of its investigation of the alleged activities of the United States in the Philippines.

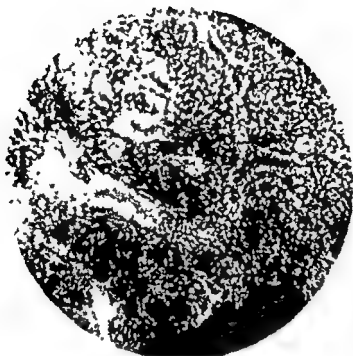


*Figs 5 and 6*

The section and the fluorogram of a 14 day old foetal mouse kidney infected with VSV and cultivated for 26 hours. The viral antigen occupies the mesenchymal condensation area but the tubuli and the mesenchyme in the middle remain unstained.

*Figs 7 and 8*

A more detailed picture of a section of foetal kidney infected with VSV and cultivated for 10 days along with the corresponding fluorogram. Note that the viral antigen appears in the cells throughout the organ but yet the collecting system and its neighbouring mesenchyme as well as the convoluted tubuli are free from antigen.





the sections were stained with fluorescent conjugate or stored at  $-40^{\circ}$ . The mounting medium was either buffered glycerol or Elvanol (Dupont).

After fluorescent microscopy, the coverslips were removed in glycerol and the sections stained with haematoxylin eosin or haematoxylin p.a.s. to reveal the structure containing the fluorescent material.

**Experimental series** 13- and 14-day-old foetal mouse kidneys were infected with polyoma virus, sectioned after 2, 4, 6 and 8 days and stained with fluorescent antibodies. Accordingly, fourteen day-old foetal mouse kidneys were infected with VSV and when they had been cultivated for 16 hours a couple of organs were removed every fourth hour and sectioned and stained with the appropriate fluorescent antiserum. The VSV studies were repeated twice, and 55 foetal kidneys were included in the experiment.

## RESULTS

**Polyoma infection** A characteristic of the infection caused by polyoma virus was that the viral antigen appeared in the loose mesenchymal tissue in the central area of an infected kidney (Vainio *et al* 1963). While the infection was advancing, the antigen appeared in the mesenchymal cells, in between the tubuli (Figs 1 and 2). However, no virus antigen was detected either in the collecting system or in the renal tubuli. Furthermore, the mesenchymal, pretubular condensations did not seem to produce viral antigen upon infection, and thus the cells forming these condensations, behaved differently from the other mesenchymal cells in the cultures.

**VSV infection** In VSV infected organ cultures, the viral antigen appeared first in the periphery. It soon occupied a rather deep tissue layer spreading inwards between the renal tubuli, which remained free of antigen (Figs 3 and 4). Within a few hours, the infected area in the periphery of cultured organ had deepened, although tubuli remained free from viral antigen (Figs 5 and 6). The area now occupied by viral antigen was rich in mesenchymal condensations, indicating their susceptibility to antigen production. When the synthesis of viral antigen could be observed in the central area of the culture, the renal tubuli appeared relatively free from antigen, as did the whole collecting system (Figs 7 and 8). It was also repeatedly noted that the mesenchyme adjacent to the collecting tubes did not seem to produce viral antigen when the infection had spread through the culture.

**Polyoma versus VSV infection** When infections caused by polyoma virus and VSV are compared, there are some obvious differences in the distribution of the viral antigen. The mesenchymal condensations (see Fig. 5 in the preceding paper) support the synthesis of VSV antigen, whereas they appear incapable of manufacturing polyoma antigen during infection. On the other hand, both collecting and convoluted tubuli appeared to be poor soil for the synthesis of these two viruses, or at least of their antigens. The undetermined mesenchyme (Fig. 5 in the previous paper) was susceptible to the synthesis of polyoma virus as well as VSV virus antigens, whereas the mesenchyme next to the collecting tubes supported the synthesis of polyoma virus antigen but appeared to be resistant to the synthesis of VSV antigen. Finally, the

spreading of the infection when caused by polyoma virus was found to be different from that caused by VSV. The former tended to start in the central portion of the culture and the latter regularly from the periphery.

### SUMMARY AND CONCLUSIONS

The results presented above show that the distribution of VSV antigen in foetal mouse kidney *in vitro* closely corresponds that of the histologically observed viral lesions. Furthermore this study supports our earlier suggestion of certain differences in the tissue competence (susceptibility) towards the two viruses (VSV and polyoma virus) employed in our studies so far. Attention was focused on changes during tubulogenesis where three morphologically distinct stages can be seen: *Undifferentiated metanephrogenic mesenchyme* → *mesenchymal condensations* → *epithelial tubules*. The mesenchyme seems to support the replication of both viruses employed whereas renal tubules appear to be resistant to these viruses. Mesenchymal condensations which represent the first detectable step in tubulogenesis resist polyoma virus but are highly susceptible to VSV and support its replication.

It may be concluded in this connection that the induction process interferes with the susceptibility of a given tissue to a particular virus. However the tissue can remain susceptible to some other viruses. Processes relevant to differentiation such as metabolic changes in the differentiating cells have a bearing on viral susceptibility. Some of these views will be discussed further in our forthcoming paper.

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## THE CYTOLOGY OF THE ADRENAL CORTEX IN MICE WITH SPONTANEOUS ADRENOCORTICAL LIPID DEPLETION<sup>1</sup>

By

KRISTEN ARNESEN

Received June 62

A spontaneously occurring lipid depletion of the adrenal cortex in mice of the AKR/O strain has been described by Arnesen (1955, 1956). The lipid depletion takes place at the time of puberty and depends upon the presence of the gonads both in males and females. Prepubertal gonadectomy prevents the lipid depletion from taking place.

In adult males the lipid depletion is almost total in terms of sudanophilia. Occasionally a narrow rim of sudanophilic material may be found in the outer, immediately subcapsular part of the cortex. In females the depletion is subtotal, and the lipid pattern of the cortex shows considerable variation.

Large doses of ACTH administered to adult AKR/O males restore the lipid concentration and distribution in the adrenal cortex to almost normal.

The adrenocortical lipid depletion is due to the effect of one recessive gene, and the following designations have been introduced: *The adrenocortical lipid pattern gene*, and *the adrenocortical lipid depletion gene*, which latter is the recessive allele, responsible for the strain specific adrenocortical lipid depletion.

The AKR/O strain exhibits a high incidence of lymphatic leukaemia. In addition to the particular constitution of the adrenal cortex, this strain is also characterized by a thymus hyperplasia which is manifest from the period of infancy. In hybridization experiments some sort of an association between the adrenocortical lipid depletion and the thymus hyperplasia was demonstrated, but the character of this association could not be elucidated.

Because of the well known effect of adrenocortical hormones upon the thymus, it was tentatively assumed that the thymus hyperplasia might disclose a state of constitutional hypofunction of the lipid-depleted adrenal cortex. It was also discussed as a hypothesis that the specific "adrenothymic constitution" might in some way be associated with the

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<sup>1</sup> The work has been supported by Grosserer & A. Stangs legat til kreftsykdommers bekjempelse.

innate disposition for lymphatic leukaemia in this strain. No definite answers have as yet been given to these questions. *Welcalf* (1960), who worked with the same strain of mice and confirmed the finding of the adrenocortical lipid depletion as well as the thymus hyperplasia, has also postulated that the adrenal cortex of these mice is hypofunctional.

To my knowledge the adrenocortical lipid depletion as a spontaneously occurring phenomenon, has only been described in the AKR strain (AKRO designating the descendent line kept in Oslo).

From the observations referred to, various important problems emanate of which two are in the centre of interest for this author.

1) Has the adrenocortical lipid depletion, as well as the associated thymus hyperplasia, anything to do with the inherited disposition to lymphatic leukaemia in the AKRO strain?

2) What is the functional implication—if any—of the adrenocortical lipid depletion?

The first question is beyond the scope of this paper. It can only be answered through time consuming hybridization experiments, which are in progress in our laboratory. A brief report of some preliminary results will be published in this journal within short time.

The second problem is under study from various angles. The adrenocortical lipid depletion as such does not permit any definite conclusion as to the functional state of the organ. The adrenocortical lipid pattern is a function of

- a) The manufacture of lipids—including steroid hormones and their precursors
- b) The storage of lipids—including steroid hormones and their precursors
- c) The output of lipids—including steroid hormones. Under conditions of homeostasis production must equal output, but neither of these is reflected in the amount stored.

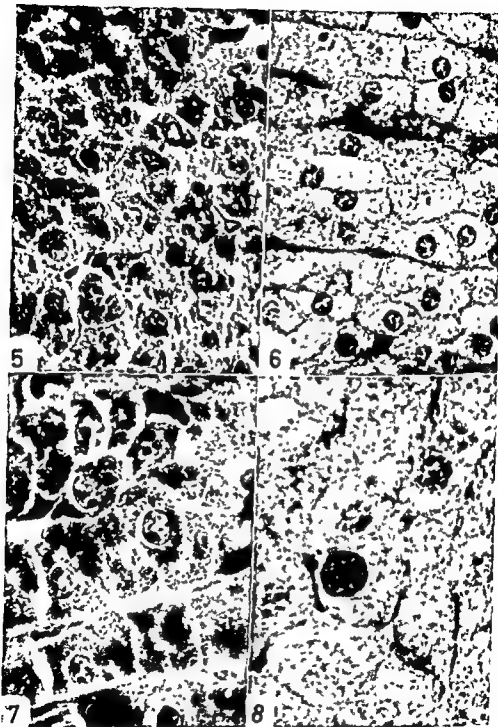
The morphological picture of permanent adrenocortical lipid depletion may imply

- a) Normal output of steroid hormones with normal rate of production but without appreciable storage
- b) Increased output of steroids with increased production and no storage
- c) Decreased output of steroids with decreased production and no storage

The only thing which can be concluded on the basis of the permanent lipid depletion is that storage is reduced or abolished. In this connection it must be pointed out that there is no marked change in the

the  
fro  
depletion

is caused by permanent adrenocortical lipid



*Figs 5-8*

*Fig 5* CS male 180 days old Cells in zona fasciculata containing numerous mitochondria Iron haematoxylin  $\times 810$

*Fig 6* WLO male 59 days old Cells in zona fasciculata with vacuolated cytoplasm Iron haematoxylin  $\times 810$

*Fig 7* Same as Fig 5  $\times 1320$

*Fig 8* Same as Fig 6  $\times 1320$

From the Figures 5 and 7 it can be seen that the cortical cells from mice with lipid depletion have a cytoplasm which is tightly packed with small round bodies with a strong affinity to the haematoxylin. These usually fill out the whole cell body to the very border of the cell. The surface membrane therefore usually appears indistinct. In contrast, the cortical cells from the normal mouse (Figs 6 and 8) are faintly stained (light cells, 'helle Zellen'), and their cytoplasm seems mostly to consist of closely adjacent vacuoles. Mitochondria are very indistinct, and the cell membrane is sharply drawn.

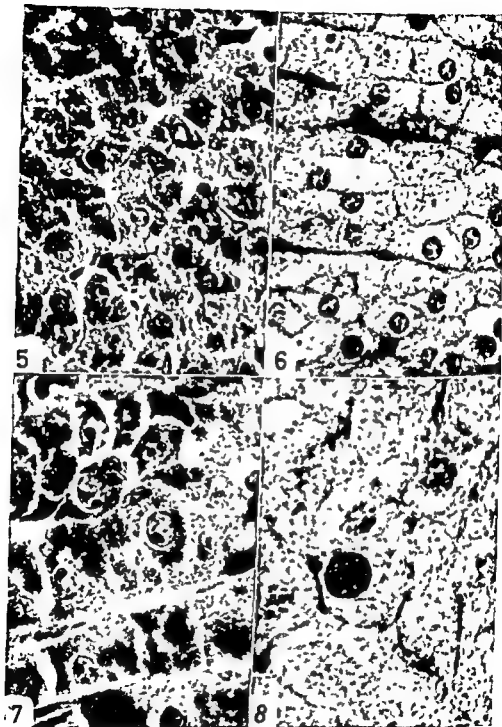
As to nuclear structure and size, no clear difference between the two types of adrenal glands have been observed, but exact measurements have not been carried out so far.

### DISCUSSION

The findings here reported should be interpreted under consideration of the work of *Molbert & Arnesen* (1960), who studied the ultrastructure of the adrenal cortical cells of normal mice, as well as those with spontaneous or artificially induced lipid depletion. In the normal adrenal cortex cells were found with large lipid vacuoles ('Speicherungslocher') between which appeared mitochondria with a rosette like structure. In the cortical cells of lipid depleted adrenals the lipid vacuoles were almost completely absent, whereas the mitochondria were abundant, closely packed, showing a characteristic pattern with double contoured *cristae mitochondriales* in a more or less parallel configuration. The present study confirms that the cortical cells in spontaneously lipid depleted adrenals are very rich in mitochondria—apparently richer than the corresponding cells of adrenals with a normal cortical lipid pattern. These findings are hardly compatible with the concept that the spontaneous lipid depletion reflects a state of permanent hypofunction of the cortex.

The term 'function' in this connection is equivocal, however. One thing is the total or partial function in terms of specific hormone production. Another is the basic and unspecific metabolic activity of the cells.

The mitochondria are of paramount importance for the oxidative processes going on in the cells, in so far as they carry the majority of the respiratory enzymes of the cytoplasm. The difference in the number and the corresponding variation in the activity of these organelles can not yet be interpreted to the endocrine function of the cortex. On the other hand, the observations favour the hypothesis that the basic metabolic (respiratory) activity is high in the lipid depleted adrenal cortical cells—probably higher than in the corresponding lipid laden cells of normal adrenal glands.



Figs 5-8

- Fig 5 CS male 180 days old Cells in zona fasciculata containing numerous mitochondria Iron haematoxylin  $\times 810$   
 Fig 6 WIO male 59 days old Cells in zona fasciculata with vacuolated cytoplasm Iron haematoxylin  $\times 810$   
 Fig 7 Same as Fig. 5  $\times 1320$   
 Fig 8 Same as Fig. 6  $\times 1320$

## THE EFFECT OF MATERIAL PREPARED FROM OX BLOOD ON COLD STRESS IN MICE

By

J. DEDICHEN, P. LALAND and S. G. LALAND

Received 19 XI 62

It has been reported that high molecular material prepared from blood increases resistance *in vivo* to certain bacterial and viral infections (1, 2). These observations indicated that we are dealing with a humoral component of importance to the so called non-specific resistance. For this reason it was thought of interest to study the effect of the material under other conditions where resistance could be measured. This report describes experiments designed to study the effect of the material in mice exposed to cold stress.

### MATERIALS AND METHODS

Two materials designated IV and VI prepared from ox blood as described previously were used (1, 2, 3).

Swiss albino male mice each weighing 15-18 gr have been used. During the experiments the animals were kept in a cold room in two wooden boxes of the same size and each divided into 15 equal square chambers. The temperature in the cold room varied from 0 to +2°C. Between experiments the empty boxes were always kept in the cold room to secure uniform temperature in all the chambers. The mice were without access to food or water during the stress test of 1-1½ h.

Animals and the controls were distributed in the two boxes at random so that both controls and treated animals were mixed. There was one mouse in each chamber and 15 treated and 15 control mice were normally used in each experiment. The number of dead animals was recorded every hour for 7 hours.

### RESULTS

The results obtained using varying amounts of material IV and VI per mouse, are seen in Fig. 1-7. The results recorded in each of these comprise a number of experiments.

Table 1 gives a survey of the statistical significance of the results recorded in Fig. 1-7. It will be seen that the material IV and VI



adrenal cortex into compact cells under the influence of ACTH or in conditions of prolonged stress, is an expression of increased cellular activity

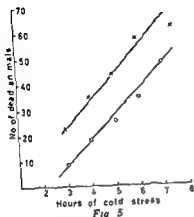
Studies of cellular metabolism as well as of the production rate and output of hormones are called for in order to reveal the real implication of the spontaneous adrenocortical lipid depletion. Investigations along these lines have been taken up in our laboratory

### SUMMARY

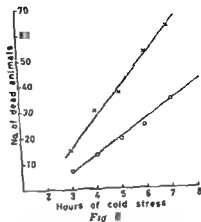
The spontaneous lipid depletion of the adrenal cortex of mice derived from the AKR/O strain, is determined by the 'adrenocortical lipid depletion gene', and is associated with structural changes in the cytoplasm of the cortical cells, characterized by loss of lipid vacuoles and the occurrence of great numbers of closely packed mitochondria. Cortical cells with a normal lipid pattern are relatively poor in mitochondria, as far as can be judged from the light-microscope pictures. The functional implication of the spontaneous lipid depletion is discussed

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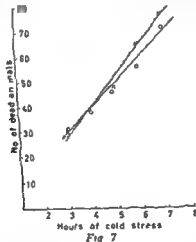
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Results of cold stress  
using 0.084 mg/mouse of material VI  
105 mice in each group  
—○— treated —X— controls



Results of cold stress  
using 0.168 mg/mouse of material VI  
105 mice in each group  
—○— treated —X— controls



Results of cold stress using 0.672 mg/mouse of material VI 90 mice in each group  
—○— treated —X— controls

With 96  $\mu$ g the effect is apparent from 5th hour and the significance increases up to seven hours

Similar results were obtained with material VI which seems less active than material IV since 42  $\mu$ g produced no protection. With 84  $\mu$ g the protective effect is apparent already after three hours, but disappears after six hours. With 168  $\mu$ g the effect starts after 4 hours and the significance increases up to seven hours where it is very marked. At 192  $\mu$ g of material IV and 672  $\mu$ g of material VI no effect was apparent.

Material IV and VI have been prepared by somewhat different procedures which probably explains the difference in activity.

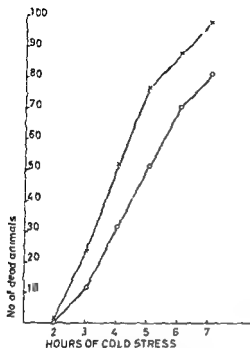


Fig 1

Results of cold stress  
using 0.018 mg/mouse of material IV  
135 mice in each group

○ — treated × — controls

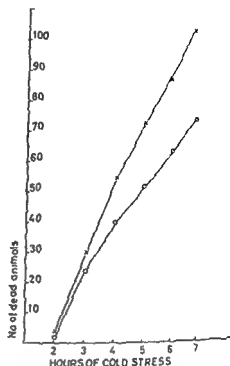


Fig 2

Results of cold stress  
using 0.096 mg/mouse of material IV  
135 mice in each group

○ — treated × — controls

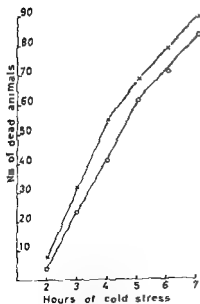


Fig 3

Results of cold stress  
using 0.192 mg/mouse of material IV.  
120 mice in each group

○ — treated × — controls

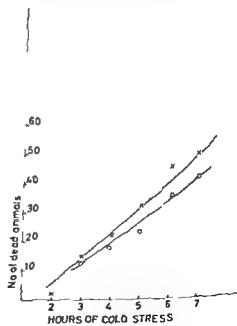


Fig 4

Results of cold stress  
using 0.042 mg/mouse of material VI  
105 mice in each group

○ — treated × — controls



TABLE 1

*Statistical Probability for the Significance of the Difference in the Number of Dead Animals in the Treated and Untreated Groups in Experiment 1-8 When P Is Greater than 0.05 no Figure Is Recorded*

Hours of cold stress	Amount of material IV injected			Amount of material VI injected				
	48 $\mu$ g	96 $\mu$ g	192 $\mu$ g	42 $\mu$ g	84 $\mu$ g	168 $\mu$ g	336 $\mu$ g*	672 $\mu$ g
3	0.030	—	—	—	0.007	—	—	—
4	0.006	—	—	—	0.006	0.003	—	—
5	0.002	0.010	—	—	0.008	0.004	—	—
6	0.007	0.003	—	—	0.001	< 0.001	—	—
7	0.012	< 0.001	—	—	—	< 0.001	—	—

\* In this experiment only 60 mice in each group were stressed

## DISCUSSION

The interest in the non-specific resistance has lately been stimulated through extensive experimental work on the endotoxins. These stimulate the resistance of the organism against infections with bacteria and viruses (4, 5, 6, 7), experimental shock and X-irradiation (8, 9).

The finding that our materials increase the resistance to cold stress over a certain range of dosage whereas higher doses do not produce any effect is interesting and surprising. It can hardly be attributed to a toxic effect of the materials which have an LD 50 estimated to 1.9 g/kg mice<sup>1</sup>. It is of interest to compare our results with the response to bacterial endotoxins, where a similar pattern is found. Thus *Greene et al* have demonstrated a dosage response relationship over a certain range in the response to treatment with Pyromen in thermally injured rats (10). A similar pattern is found when the migration of leukocytes after intraperitoneal injection of endotoxin in mice is estimated (11). Since the materials are not pyrogenic in rabbit in 5 mg dose (2) the effect of our material from blood is hardly due to contaminating pyrogens.

The mechanism which leads to the death of the animal during cold stress is probably complex. *Selye* (12) has pointed out that cold stress represents an ideal example of alarm reaction with haemoconcentration, hypoglycaemia and intestinal haemorrhages, reactions which are not obscured by reactions due to the shock-producing stimulant itself. The experiments reported here were not carried out to investigate the mechanism of cold stress, and no detailed examination of our animals

1 Unpublished results

## ETHYLENE OXIDE STERILIZATION WITHOUT SPECIAL EQUIPMENT<sup>1</sup>

By

KERSTIN WINGE HEDÉN

Received 11 xli 62

In the prevention of nosocomial infections, problems of sterilizing hospital equipment are important. Most types of endoscopes, often with delicate and expensive optics, have long presented difficulties as regards sterilization. More and more refined instruments with complicated electrical parts are coming into use in hospitals, as are many varieties of plastics (Engley 1951, Grundy, Rdzok, Remo, Sagen & Sylvester 1957). High temperatures and steam usually have deleterious effects on such instruments and materials. Satisfactory sterilization is, however, necessary and this is why gases such as ethylene oxide have increasingly been used as sterilizing agents in the past decade (Bekker & Onulee 1956, Davis, Wolkoff & Leonards 1957, Diding & Kjellman 1958, Skeehan 1959, Thomas 1960, Bracken, Wilton-Davies, Weale & Kelsey 1960, Kelsey 1961). Since the potentialities of gas sterilization nevertheless are not fully utilized in many sectors of hospital routine, a review of the general properties and uses of ethylene oxide would seem to be warranted.

### General Properties

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*Arch int pharmacodyn* 127 190 1960
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*mittel Forschung* 11 825 1961

centrations over fairly long periods can produce nausea vomiting headache and dizziness (Blackwood & Erskine 1938) Pulmonary oedema may follow exposure to high concentrations of the gas Gaseous ethylene oxide in a concentration of about 6 mg per liter can be tolerated for an inhalation period of at most 60 minutes

Liquid ethylene oxide as a rule evaporates from the skin so rapidly that cooling is the only result More protracted contact between the skin and substances in which the gas has been dissolved (rubber leather plastic etc.) may give rise to blistering, however (Royce & Moore 1955) This has been observed when for instance, laboratory staff used rubber shoes which shortly before had been sterilized with ethylene oxide (Phillips 1958) Sensitization to ethylene oxide has been experimentally evoked (Sexton & Henson 1949 and 1950) Hypersensitivity reactions, however are rare

### Practical Use

Sterilization with ethylene oxide is dependent upon the following factors exposure time, concentration (partial pressure of ethylene oxide in the chamber), temperature and humidity In most cases the recommended concentration of ethylene oxide is 450 mg per liter for 5 hours at a temperature of about 55° C If the concentration can be raised to 1000 mg per liter, the exposure time can be reduced to c 3 hours In order to reach such concentrations, the exposure must take place at pressures of 2 to 6 kg per cm<sup>2</sup> when 10 per cent ethylene oxide in CO<sub>2</sub> is used and at 1 to 2 kg per cm<sup>2</sup> when 20 per cent ethylene oxide is used For production of these conditions and the necessary temperature and humidity, special apparatus has been employed as a rule Several autoclave like designs are now on the market, for instance American (American Sterilizer Co Carboxide, Cryoxide, Oxyfume equipment) German (DMB Sterovit equipment) and Finnish (Oy Santasalo-Sohlberg Freoxide equipment)

By using a mixture of 11 per cent ethylene oxide and 89 per cent fluorinated hydrocarbons (freon) it has been found possible to effect sterilization at normal atmospheric pressure Thus, Schley, Hoffman & Phillips (1960) published a method for sterilizing clothes, etc by placing them in polythene bags together with a container of ethylene oxide-freon The container was punctured and the gas escaped into the bag, where it gave a concentration of about 500 mg per liter Like many other plastics however, the bags used in these experiments were permeable to the gas (Waack, Alex Frisch & Schwarc 1955) and, as a result, exposure times and concentrations could hardly be reliably controlled

The aims of the present study, based on the desirability of a simple gas sterilization method suitable for routine use, were as follows

- 1 To find a material suitable for making sterilizing bags and having the following properties



these substances. An important point is that ethylene oxide is active even in the presence of large amounts of organic substances. It also penetrates through shallow layers of oil or water, which are thereby sterilized. Ethylene oxide rapidly disappears after use, but relatively long airing is advisable after sterilization of thick or dense materials, for instance rubber or plastic, in which it may have dissolved (Phillips & Warshawsky 1958).

In water ethylene oxide is hydrolyzed to ethylene glycol, which has considerably lower sterilizing potency. At room temperature this reaction takes place slowly but it is accelerated by various catalysts, particularly mineral acids and some metals such as copper.

TABLE 1  
*Ethylene Oxide Mixtures for Sterilization*

Commercial name	Maker	Ethylene oxide (%)	Inert gas (%)	Properties	Sterilization pressure
T-gas	Deut. Gesellsch. f. Schädlingsbekämpfung mbH Frankfurt a/M	90	10 CO <sub>2</sub>	explosive	
Etox	London Fumigation Co. Ltd. London				
Oxysume	Union Carbide & Carbon Corp. New York 17 N.Y.	20	80 CO <sub>2</sub>	explosive	1 atm
Alto	Sterisist GmbH Mainz a/Rh.	12-15	88-85 CO <sub>2</sub>	nonexplosive	4-7 atm
Cartox	Deut. Gesellsch. f. Schädlingsbekämpfung mbH Frankfurt a/M	10	90 CO <sub>2</sub>	nonexplosive	1.29 atm
Carboxide	Union Carbide & Carbon Corp. New York 17 N.Y.	10	90 CO <sub>2</sub>	nonexplosive	1.29 atm
Cryoxide	American Sterilizer Co. Erie Penn. U.S.A.	11	44% CCl <sub>2</sub> F <sub>2</sub> 44% CCl <sub>3</sub> F	nonexplosive	1.35-2.2 atm
Freoxid	Oy. Santasalo Sohlberg Helsinki	10	90 Freon	nonexplosive	1.28 atm

Ethylene oxide is highly explosive in air even in concentrations as low as 3 per cent (Hess & Tilton 1950). For sterilization in hospitals therefore nonexplosive and nonflammable mixtures are usually employed (Haenni, Affens, Lento, Yeomans & Fulton 1959). Table 1 presents data concerning some mixtures. It is based largely on a report by Bruhin, Bühlmann, Vischer & Jammers (1961). Fluorinated hydrocarbons ethylene oxide mixtures are rapidly effective even at ordinary atmospheric pressure. As compared with corresponding mixtures of ethylene oxide and carbon dioxide they have lower vapour pressure and contain more ethylene oxide per unit of volume.

(Waller & Greeson 1932; Amdur & Meadter & Spencer 1956; Jacobson, Hackley & ...). Ethylene oxide vapour irritates the respiratory tract in much the same way as ammonia, itching and burning sensations may occur in the eyes. The gas is more toxic to small than to large animals. Inhalation of low con-



Fig 1.

filled. After some practice this can be reduced to a single extra spraying. The bag can thereafter be stored at room temperature. The bags should be opened on a balcony or by an open window or under a hood, since the gas has some toxic properties.

Liquid ethylene oxide can cause changes in the shape of or cracks in some plastics (Spencer & Bahnsen 1958). It is therefore important that the outflow of the small tins that are placed in sterilizing bags should not be directed towards delicate material. The mouthpiece can for instance be covered with cottonwool or gauze in which the liquid can vaporize.

#### B Sterilization in Commercial Ethylene Oxide Equipments

Two commercial ethylene oxide sterilizers have been tested. The makers recommend a sterilization temperature of 55° C, but with both types of apparatus other temperatures can be used.

One apparatus (DMB-Sterisit) was run at a pressure of 6.4 kg per cm<sup>2</sup> and the recommended sterilization time was 1 hour (Gewalt & Fischer 1959). A mixture of 15 per cent ethylene oxide and 85 per cent CO<sub>2</sub> was used. Humidity was obtained with the aid of a piece of elastic sponge which was moistened before use.

The other apparatus (DMB-Sterisit) was run at a pressure of 10 kg per cm<sup>2</sup> and 90 per cent humidity was maintained. A small amount (1 ml) of water was added after a vacuum had been created. With regard to sterilization times, the makers provided the following table:

Pressure (kg cm <sup>2</sup> )	Concentration of ethylene oxide (mg liter)	Exposure time (hours)	
		40° C	55° C
0.5	600	4-6	3-5
1.0	800	3-5	2-4
1.5	1000	2-4	1½-3
2.0	1200	1½-3	1-2

- a) impermeability to ethylene oxide,
  - b) strength, and
  - c) ease of sealing
- 2 To study the efficiency of this system
  - 3 To compare it with conventional ethylene oxide sterilizers and with a standard hospital autoclave converted by simple measures into an ethylene oxide sterilizer

A brief preliminary report has been published in the form of an abstract (*Winge-Heden 1962*)

## METHODS

Three methods of ethylene oxide sterilization were used

- A In three foil bags
- B In commercial ethylene oxide sterilizers
- C In a converted hospital autoclave

### A Sterilization in Three Foil Bags

**Material** Since the majority of plastics are more or less permeable to ethylene oxide plastic alone was not considered suitable for making sterilizing bags. A material—three foil—made by Åkerlund & Rausing<sup>1</sup> was found to be suitable however and was kindly made available to me. The foil was supplied in rolls of varying widths. As its name implies it has three layers. The outermost layer is of fairly strong but flexible paper. Beneath this is a layer of aluminium foil and innermost a thin layer of polythene plastic. The three layers are closely adherent to each other and can only be separated with difficulty. This renders the material tough and strong. It is highly resistant to handling and moisture and the paper surface is suitable for notations. The aluminium foil makes the material gas impermeable. Because of the plastic lining the bags are extremely simple to make since all that is required is to place two plastic layers in apposition and then run over the material with a hot household iron. A fairly broad seam can thus be obtained and through this thin and broad plastic barrier no significant diffusion of gas occurs as indicated by the observation that the gas remained in bags stored as long as 4 months.

The ethylene oxide from Freon<sup>2</sup> sterilization in these bags can be released by a valve or by puncture of the tin. In my experiments the gas was supplied by a Finnish firm (Oy Santasalo Sohlberg). Three sizes of tin are available (containing 0.1, 0.4 and 0.6 kg gas mixture i.e. 10 per cent ethylene oxide and 90 per cent freon). The gas in the tins is under pressure and is sprayed out through a small valve which can be manipulated from outside the bag.

**Making three foil sterilizing bags and technique of sterilization** For each bag about 60 × 100 cm of three foil were used. The material was folded double with the plastic surfaces against each other. Three sides of the bag were then sealed with the aid of a hot iron. The bacteriological test pack (see below), an 0.1 kg tin of freon, ethylene oxide and a small moistened swab of cottonwool or a moist piece of filter paper were placed in the bag as shown in Fig. 1. As much as possible of the air in the bag was pressed out before the fourth side was sealed by ironing. In this connection articles to be sterilized preferably should not be forced out. For such articles larger bags should be used. The gas is released without difficulty, felt from outside the bag. Spraying is interrupted after a few seconds while the operator observes how much the bag expands when the liquid is vapourized. Spraying and pause are repeated until the bag is

<sup>1</sup> Åkerlund & Rausing Ltd, Lund, Sweden. Paper 70 g/m<sup>2</sup> + 11.09 mm + polythene 45 g/m. Total weight 140 g/m<sup>2</sup>.

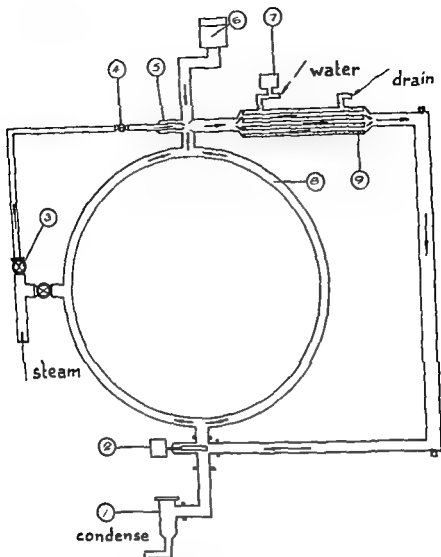


Fig. 2

- 1 Condensate trap
- 2 Sensing element for temperature
- 3 Valve
- 4 Fine adjustment valve
- 5 Ejector
- 6 One way air valve opened by vacuum in jacket
- 7 Magnetic valve
- 8 Steam jacket
- 9 Cold water cooler

valve which hitherto had been in the "closed" position. The pressure in the jacket was set to 2 until the pressure in the door screws were further setting the central valve to k only a few seconds. After

### C Sterilization in a Converted Steam Autoclave

At the research department of the Clinic for Thoracic Surgery an ordinary horizontal hospital autoclave (Geringe, type SAL 8), jacketed and provided with a central valve, volume c. 500 liters, was modified so that it could be used alternatively for ethylene oxide or steam. Such modifications have been tested by other workers (Kaye 1950, Skeechn, King & Kaye 1956, Spencer & Bahnson 1958, Sager & Iken 1959, Schley, Hoffman & Phillips 1960, Freeman & Barwell 1960). The arrangement here selected, however, was found to be simple and cheap as well as extremely reliable. For these reasons some details of the construction of the apparatus, worked out in collaboration with Geringeverken Ltd., are now presented.

The central valve of the autoclave controls the following connection (O = open, C = closed):

- a air intake—autoclave chamber,
- b. steam from mantle to chamber,
- c. steam from chamber to exhaust, and
- d autoclave chamber to vacuum pump

The valve has 6 positions:

- 1. Stop, preheating (a/O, b/C, c/C, d/C).
- 2. Vacuum 65 cm Hg (a/C, b/C, c/C, d/O).
- 3. Pressure rise 2.0–2.5 atmospheres (a/C, b/O, c/C, d/C).
- 4. Sterilization (a/C, b/O, c/O, d/C).
- 5. Steam removal (a/C, b/C, c/C, d/O).
- 6. Drying (a/O, b/C, c/C, d/O).

The air intake of the autoclave is fitted with a small filter. In the conversion to an alternative ethylene oxide sterilizer, a three-way cock was inserted between the autoclave and the filter. By means of this cock the autoclave chamber or, more correctly, the central valve could be connected with the filter or, via a reinforced tube with a "Cartox" cylinder which was provided with a pressure-reducing valve. The filled cylinder weighed approximately 85 kg. It contained about 25 kg of gas mixture and was suspended in a spring balance (Salter No 235, can weigh 100 kg with an accuracy of 0.5 kg). This arrangement was necessitated by the inadvisability of using more than about 80 per cent of the gas mixture, since otherwise there is a risk that the concentration of  $\text{CO}_2$  will become excessive.

An expanding gaseous mixture chills rapidly, and therefore it is desirable that the pressure-reducing valve, which is left open throughout the sterilizing phase, should be of modern heatable type. In addition, the communication between the central valve and the autoclave chamber must partly be steam jacketed as a protection against condensation in bends and couplings. It must also be provided with a safety valve adjusted to 5 kg per  $\text{cm}^2$  and connected to the steam blow off pipe of the apparatus. Another safety valve, set at 3 kg per  $\text{cm}^2$ , can open the tube between the central valve and the autoclave chamber to the steam blow off pipe when the pressure exceeds that level. Otherwise no changes are required apart from those for thermoregulation. The water ring pump for production of vacuum may be used for instance, since the gas is highly soluble in water. The pump, however, should be placed in a relatively well-ventilated site.

A simple thermoregulating system, which does not interfere with the normal steam sterilizer function was designed as shown in Fig. 2. The thermostat (2) directs the flow of cold water through the magnetic valve (7), so that the steam sprayed through the ejector (5) is cooled. A valve (6) draws in a fixed volume of air which is mixed with the steam leaving the jacket (8) of the autoclave. To the intake of the jacket at (2) flows a mixture of steam, air and condensate which maintains the desired temperature of the thermostat. The condensate is removed through a trap (1) and the air and steam mixture flows through the jacket and heats the autoclave.

The procedure for ethylene oxide sterilization with this apparatus was as follows. The main steam valve and the valves to the gas heater and the jacket thermoregulation (No. 3 in Fig. 2) were opened. The electrical thermostat, set at  $+60^\circ\text{C}$ , was activated. The nonsterile material was placed in the apparatus and the door was carefully closed. The gas cylinder was lifted up in the spring balance, its weight was noted and the balance again unloaded. The three-way cock at the air filter was closed and also the escape valve for condensate from the autoclave chamber. The central

med centrifuge tubes, in polythene foil bags (0.05–0.08 mm) or in nylon foil bags (0.05 mm thick)

In the sterilizers in which release of the gas was preceded by a vacuum phase, it was necessary to tie a single layer of gauze over open tubes and dishes to prevent the light test foil from being whirled up. The same procedure was also used for sterilization in three foil bags, since otherwise there was risk that the test slips might fall out of the dishes when the bags were handled

* Sterile samples	Number of samples	Exposure humidity source	Enclosure of samples	Time, hrs	Temp °C
50	100				
—	33	One water drop on each sample	Open petridishes	6	22
—	26				
—	27				
—	32		Nylon bags	6	22
—	30				
—	36				
—	20	Moist cotton swab in the nylon bags	Nylon bags	6	22
—	3				
—	13		Nylon bags	6	22
—	13				
—	18	Moist cotton swab in the nylon bags	Nylon bags	6	37
—	20				
—	19				
—	67	One water drop on each sample	Nylon bags	18	22
—	20				
—	17				
—	10		Open test tubes length about 10 cm	18	22
—	5				
—	10				
—	63	Moist cotton swab in the nylon bags	Nylon bags	18	22
—	24				
—	19				
—	13				
—	8		Nylon bags	18	22
—	13				
—	22				
—	37		Petridishes with lid	18	22
—	36				

Fig 3  
Three foil bags  
Samples Aluminium foils charged with

— spores of *B. brevis*  
 - - - *Staph. aureus*  
 — *Str. faecalis*

this the valve was adjusted to 1 and the sterilizing gas was turned on the pressure reducing valve set to 2 atmospheres. Six hours after this pressure had been reached which took about 15 minutes, the flow of gas was turned off and the material was aired by setting the central valve to 6. When the pressure had fallen to atmospheric level, the aircock was opened. About 15 minutes later the central valve was again turned to 1 and the initially described steps were repeated in reverse.

The gas consumption per run was 2 to 3 kg. Small leaks in valves and elsewhere produced minor variations in this respect. Since the gas also diffuses out through rubber gaskets etc. the cylinder should be turned on throughout the time of sterilization.

### *Bacteriological Investigation Technique*

The efficiency of all the described methods was tested by determination of the bactericidal potency with respect to

- 1) spores (of *Bacillus brevis*), and
- 2) vegetative bacteria (*Staphylococcus aureus* 209 Oxford, and a local strain of *Streptococcus faecalis*).

The *Bacillus brevis* strain was obtained from the bacteriological department of Karolinska Institute, where it was used for a simplified autoclave test (Heden & Markula 1962) requiring micro-organisms with high heat resistance.

Suspensions of these micro-organisms were carefully dried on punched-out rounds of filter paper 10 mm in diameter (Schleicher & Schüll no. 2208) or on 0.015 mm thick squares of aluminium foil. Micro-organisms dried on metal foil present a considerably more exacting test of ethylene oxide sterilization than do micro-organisms on porous material. When this difference had been confirmed in all the tested types of apparatus, therefore, I ceased to use filter paper and confined the tests to the pieces of aluminium foil.

TABLE 2  
*Data Concerning the Tested Sterilization Methods*

Type of apparatus	Gas mixture		Time hours	Temperature °C	Pressure kg cm <sup>2</sup>
	ethylene oxide %	inert gas %			
Commercial high pressure sterilizer	15	85	1	55	6.4
Commercial low pressure sterilizer	10	90	4-6	55	2.4
Converted autoclave	10	90	6	60	2.0
Sterilizing bags	10	90	6-18	22	1.0

### *Wrapping Technique*

In order to study the influence of wrapping the articles on the results of ethylene oxide sterilization, the placement of the test foil was varied, for instance in open or closed Petri dishes, in open and cottonwool-plug-

* Sterile samples	Number of samples	External humidity source	Enclosure of samples	Time in hrs	Temp		
0                      50                      100	20	One water drop on each sample	Open petridishes	6	55		
-----	20			4	50		
-----	20						
-----	21			4	50		
-----	15	-----	Open test tubes horizontal				
-----	15						
-----	15						
	10	-----	Open test tubes horizontal	6	55		
	10						
	10						
	5	-----	Petridishes with lid	4	50		
	5						
	5						
-----	10	Moist cotton swab in the polyethylene bags	Polyethylene bags	4	50		
-----	5						
-----	5						
-----	15						
-----	14	Moist cotton swab in the nylon bags	Nylon bags	6	55		
-----	15						
-----	5						
-----	5						
-----	5	Moist cotton swab in the nylon bags	Nylon bags	16	55		
-----	9						
-----	9						
-----	9						
-----	3	Moist cotton swab in the nylon bags	Nylon bags	6	55		
-----	3						
-----	3						
-----	3						

Fig 5

Low pressure ethyleneoxide sterilizer  
Samples Aluminum foils charged with

----- spores of *B. brevis*  
----- *Staph aureus*  
----- *Str faecalis*

Variations in sterilization time, temperature, pressure and gas mixture in the different experiments are shown in Table 2

## RESULTS

The results of the experiments are presented in Figures 3-8. A few bizarre results occurred. For instance, the effect was sometimes better after short than after longer exposure to sterilizing gas, but most of these deviations occurred with the commercial low pressure apparatus.



Humidity can be of decisive importance in ethylene oxide sterilization (Phillips & Kaye 1949). Tests were therefore made with dry aluminium squares and with squares which had been moistened with a small drop of water. When dry pieces of foil were packed in nylon or polythene bags, a moist swab was as a rule also inserted, but in other tests the foils were packed dry. In studies of the commercial sterilizers, the maker's recommendations with regard to humidity were always followed.

% Sterile samples			Number of samples	Extra humidity source	Enclosure of samples	Time in hrs	Temp °C
			Samples    Aluminium foils				
0	50	100	14	One water drop on each sample	Open petridishes	1	55
-----		20					
-----		18					
-----		45	-----		Open test tubes horizontal	1	55
-----		20					
-----		20					
-----		38	-----		Petridishes with lid	1	55
-----		10					
-----		10					
-----		20	-----		Polyethylene bags	1	55
-----		20					
-----		20					
-----		15	Moist cotton swab in the polyethylene bags	Nylon bags	Nylon bags	1	55
-----		17					
-----		13					
-----		20	Moist cotton swab in the nylon bags		Nylon bags	1	55
-----		19					
-----		20					
-----		20	-----		Nylon bags	1	55
-----		5					
-----		5					
			Samples    Filterpaperdiscs				
-----		22	-----		Open petridishes	1	55
-----		23					
-----		26					
-----		38	-----		Open test tubes vertical	1	55
-----		38					
-----		38					
-----		20	-----		Nylon bags	1	55
-----		20					
-----		20					

Fig 4

High pressure ethyleneoxide sterilizer  
 Samples    Test slips charged with  
 ----- spores of *B. brevis*  
 ----- *Staph. aureus*  
 ----- *Str. faecalis*

* Sterile samples			Extra humidity source	Enclosure of samples	Time in hrs	Temp °C
0	50	100				
_____	20	} One water drop on each sample	}	Open petridishes	6	55
_____	20					
_____	20				4	55
_____	24					
_____	15	}	}	Open test tubes horizontal	4	55
_____	15					
_____	15					
_____	10	}	}	Petridishes with lid		
_____	10				6	55
_____	10					
_____	5	}	}	Polyethylene bags	4	55
_____	5					
_____	5					
_____	10	} Moist cotton swab in the polyethylene bags	}	Nylon bags	6	55
_____	5					
_____	5					
_____	15					
_____	14	}	}		16	55
_____	15					
_____	5					
_____	5	} Moist cotton swab in the nylon bags	}		6	55
_____	5					
_____	9					
_____	9	}	}		4	55
_____	3					
_____	3					
_____	2	}	}			
_____	3					
_____	2					

Fig 5

Low pressure ethyleneoxide sterilizer  
Samples Aluminum foils charged with

\_\_\_\_\_ spores of *B. brevis*  
 - - - - - *Staph. aureus*  
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Variations in sterilization time, temperature, pressure and gas mixture in the different experiments are shown in Table 2

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I shall return to them in the discussion From the results, some general conclusions could be drawn

* Sterile samples			Number of samples	Extra humidity source	Enclosure of samples	Time in hrs	Temp °C
0	50	100					
—————	—————	—————	15	One water drop on each sample	Open petridishes	6	60
-----	-----	-----	15				
-----	-----	-----	15				
—————	—————	—————	20		Nylon bags	8	60
-----	-----	-----	20				
-----	-----	-----	20				
—————	—————	—————	15	—————	Open petridishes	6	60
-----	-----	-----	15				
-----	-----	-----	15				
—————	—————	—————	28	—————	Open test tubes length about 10 cm horizontal	6	60
-----	-----	-----	25				
-----	-----	-----	30				
—————	—————	—————	10	—————	Open test tubes vertical	6	60
-----	-----	-----	10				
-----	-----	-----	10				
—————	—————	—————	18	—————	Petridishes with lid	6	60
-----	-----	-----	19				
-----	-----	-----	20				
—————	—————	—————	25	Moist cotton swab in the nylon bags	Nylon bags	6	60
-----	-----	-----	24				
-----	-----	-----	25				
—————	—————	—————	13	—————	Nylon bags	6	60
-----	-----	-----	10				
-----	-----	-----	10				

Fig 6

Modified steam sterilizer  
 Samples Aluminium foils charged with  
 ————— spores of *B brevis*  
 ----- *Staph aureus*  
 - - - *Str faecalis*

One hundred per cent sterility was obtained with all methods (excluding the low-pressure apparatus) if the test slips

- 1) were kept in open dishes during sterilization, and
- 2) were moistened prior to sterilization

## DISCUSSION

As Figures 3 to 8 show, wide variations were seen in the results The factors, apart from temperature, time and pressure, which influenced sterilizing efficiency were

*. Sterile san pla	Num ber of san p les	1 steri and liv source	Inclosure of samples	Time in hrs	Temp °C	MOBILE D D STERILIZING
0	50	100				
	8	Moist cotton swab in the polyethylene bags	Bottom of cottonplugged horizontal testtubes in polyethylene bags	6	60	Samples Aluminumfoils charged with — Spores of B brevis — Staph aureus
	8	—	Bottom of cottonplugged horizontal testtubes in polyethylene bags	6	60	
	21 21	Moist cotton swab in the polyethylene bags	Bottom of cottonplugged horizontal testtubes in polyethylene bags	6	60	Samples filterpaperdiscs charged with — Spores of B brevis — Staph aureus
	21 21	—	Bottom of cottonplugged horizontal testtubes in polyethylene bags	6	60	
	31 30	—	Bottom of cottonplugged horizontal testtubes in polyethylene bags	1	55	HIGH J IN SUCU T TING / T N I O N I D I S T E R I L I Z I N G Samples filterpaperdiscs charged with — Spores of B brevis — Staph aureus
	29 31	Moist cotton swab in the polyethylene bags	Bottom of cottonplugged horizontal testtubes in polyethylene bags	1	61	

Fig 7

I shall return to them in the discussion From the results, some general conclusions could be drawn

% Sterile samples	Number of samples	Extra humidity source	Enclosure of samples	Time in hrs	Temp °C
0                  50                  100					
_____	15	One water drop on each sample	Open petridishes	6	60
-----	15				
-----	15				
_____	20		Nylon bags	11	60
-----	20				
-----	20				
_____	15	—	Open petridishes	11	60
-----	15				
-----	15				
_____	28	—	Open test tubes length about 10 cm horizontal	6	60
-----	25				
-----	30				
_____	10	—	Open test tubes vertical	6	60
-----	10				
-----	10				
_____	18	—	Petridishes with lid	6	60
-----	19				
-----	20				
_____	25	Moist cotton swab in the nylon bags	Nylon bags	6	60
-----	24				
-----	25				
_____	13	—	Nylon bags	11	60
-----	10				
-----	10				

Fig 6

Modified steam sterilizer  
Samples Aluminium foils charged with

\_\_\_\_\_ spores of *B. brevis*  
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## DISCUSSION

As Figures 3 to 8 show, wide variations were seen in the results. The factors, apart from temperature, time and pressure, which influenced sterilizing efficiency were

aluminium foil was used as test material Table 3 illustrates the difficulty of sterilizing aluminium foil as compared with filter paper. The 45 spore bearing aluminium foil slips and the 25 spore bearing filter paper discs were exposed to ethylene oxide for one hour at 50° C. Of the former only 11 were rendered sterile whereas all the filter paper discs were sterilized.

### Humidity

The crucial importance of moisture for ethylene oxide sterilization has already been mentioned. Phillips (1949) stated the optimal relative humidity to be about 30 per cent. In a more recent report (1961) he showed that desiccated spores and vegetative bacteria can become totally resistant to ethylene oxide. This phenomenon does not affect all the microbes in a suspension but only a minority. In the technique which I used even a very small number of surviving micro organisms show up. Phillips further reported that after direct moistening with water the resistant bacteria reassumed their usual sensitivity. Attempts to condition the desiccated microbes in high relative humidity showed that they must be left in the moist atmosphere for several days (4 days at 98 per cent relative humidity and 6 days at 78 per cent) before normal sensitivity to ethylene oxide was regained.

The possibility that a very few of the test micro organisms were so intensively dried that they became resistant to ethylene oxide presumably explains some of the apparently paradoxical results in my experiments. The test slips often were prepared in fairly large batches which were gradually used up. This meant that in some experiments the slips had been dried for only 24 hours but in other experiments the slips could have been stored dry for 2 to 3 weeks before use. The atmospheric humidity in the laboratory was very low (c. 13 to 15 per cent) for long periods (measured with the aid of Phillips Air Humidity indicator PR 9650).

Figure 1 shows that freshly prepared test slips were appreciably easier to sterilize than slips which had been stored dry for some length of time.

When the test slips were placed in plastic foil bags simultaneous insertion of a moist cottonwool swab considerably improved the results. But the moist swab clearly was not always adequate if the slips were very dry. The time during which the very dry slips lay in the humid atmosphere was then probably too short. Such an effect was specially noticeable with the high pressure sterilizer which used a very short sterilization time. Considerably better results were also obtained in the experiments with three foil bags in which the longest sterilization times were used. But here too 100 per cent sterility might not result when very dry slips were tested.

From the purely practical point of view these observations cannot be

- a) the nature of the nonsterile test material (foil or paper),  
 b) humidity, and  
 c) wrapping of the test material

% Sterile samples			Number of samples	Extra humidity source	Enclosure of samples	Time in hrs	Temp °C	
0	50	100	10	—	Nylon bags	24	22*	
-----	-----	-----	10					
-----	-----	-----	10					
-----	-----	-----	6	---		24	37§	
-----	-----	-----	6					
-----	-----	-----	6					

Fig 3

Three foil bags  
 Samples Aluminium foils charged with  
 ——— spores of *B. brevis*  
 ----- *Staph. aureus*  
 ----- *Str. faecalis*

\* Samples prepared the day before test

§ Samples prepared two months before test Low Humidity during storage

TABLE 3  
 Comparison of Sterilization Results on Spore-Bearing Test Materials

Type of test slip	No. of test slips		% sterile
	sterile	tested	
Aluminium foil	6	45	15
Filter paper	25	25	100

Sterilized in open Petridishes for 1 hr Temp 55° C  
 Pressure 6.4 atm No extra humidity

### Nature of the Nonsterile Test Material

As mentioned, pieces of aluminium foil with dried micro-organisms presented a much more exacting test of sterilizing efficiency than did porous paper. The main reason for this higher resistance to ethylene oxide would seem to be the impermeability of aluminium foil to the gas, in contrast to filter paper. During desiccation, moreover, a protective film of salt crystals forms around the micro-organisms (Mathews & Hofstad 1953, Phillips & Kaye 1949).

Many of the instruments which can with advantage be sterilized by ethylene oxide are made of polished metal or glass, both of course impermeable to the gas. In the later part of the study, therefore, only

is opened. As mentioned, extraction of sterilized articles should be done either in the open air or under a closed hood so as to avoid inhalation of the escaping gas.

Bulky objects containing large volumes of air that cannot be manually forced out are not suitable for sterilization in three foil bags of the normal size since the trapped air will render the ethylene oxide concentration inadequate. For such articles gas sterilization should be preceded by a vacuum phase.

Sterilization in three foil bags is an extremely simple method which gives good results without any special apparatus. The cost of a bag which holds about 12 liters is approximately 1 Sw. crown. The gas for this operation costs about 6 Sw. crowns.

The two commercial sterilizers gave widely varying results. With the low pressure apparatus the variation was too great to permit reliable assessment. With the high pressure apparatus 100 per cent sterility was obtained when the test slips of aluminium lay in open dishes and were moistened with a drop of water before sterilization. The tests with filter paper discs gave considerably better results. Metal or glass articles thus should not be placed in closed boxes or wrapped in foil before sterilization in such commercial apparatus. Nor should dipping of the instruments in water be omitted before sterilization. The brevity of the exposure periods probably explains the comparatively poor results with wrapped test slips. In brief ethylene oxide exposure the gas does not have time to diffuse through foil wrappings or to become distributed in pockets of air that can exist in tubes or dishes. When closed Petri dishes are used the high pressure may compress the lid against the edge of the dish thus delaying diffusion of the gas. Brief exposure further implies that desiccated micro organisms are not adequately moistened even when

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The autoclave converted for ethylene oxide sterilization like the high pressure commercial sterilizers gave satisfactory results in tests using aluminium foil slips which had been moistened with a drop of water and were exposed in open dishes. In addition test slips wrapped in nylon foil together with a moist cottonwool swab were sterilized in all but one test. The preliminary vacuum phase and the longer exposure to ethylene oxide undoubtedly explain the superior results as compared with those from the high pressure commercial autoclave. The importance of the vacuum phase was also demonstrated by the experiments in which the test slip was placed in a cotton plugged test tube and then in a bag of polythene foil. With this arrangement the converted autoclave was clearly superior to the high pressure commercial autoclave. A pertinent point is that bags containing bulky articles should not be



ignored. Materials which are to be sterilized after very dry storage for some length of time thus should preferably be dipped in water prior to ethylene oxide sterilization, or should be packed together with a source of moisture.

By comparison with other bacteriological studies of the sterilizing action of ethylene oxide, some of my experiments may seem to have given poorer results. But scrutiny of other writers' reports shows that in many cases their test material was dried only for an hour or two and sometimes not at all, which appreciably facilitated sterilization. In all my experiments the test slips had been dried for at least 24 hours and often for appreciably longer periods. Since they therefore constituted a very severe test, any good results must be considered to have a wide margin of safety which, of course, is desirable in all sterilizing of hospital material.

### *Wrapping of the Nonsterile Material*

It was to be expected that the best results of ethylene oxide sterilization should be obtained when the test material lay open. In hospital work, however, wrapping of the various articles, preferably in bacteria proof foil, is frequently necessary for ease of handling, transportation and storage and to minimize risk of contamination. Nylon film, polyvinyl chloride and polythene are widely used as wrappings for both steam and ethylene oxide sterilization.

In my experiments nylon foil was found to be a highly suitable wrapping, but polythene foil also gave good results. In the high-pressure apparatus the latter gave better results, but detailed comparisons of the various plastic foils were not made.

If it is desired to sterilize articles in boxes or trays, these should be left open and sterilization should preferably be done in an apparatus which permits a preliminary vacuum phase.

*Sterilization in three-foil bags* gave results which were equivalent to and often better than those where commercial sterilizers or the converted autoclave were used. The superior effect probably was attributable to the longer sterilization times. With the three-foil method, for instance, dry aluminium foil slips in closed dishes were rendered sterile, which could not always be achieved by the other methods. The recommendations for wrapping of the nonsterile articles must be observed, however, which implies that if these articles are packed in nylon, etc. a moist cottonwool swab or filter paper must be inserted under the wrapping. The articles can also be placed directly in the three-foil bag, when it is important not to omit the moist swab in the bag.

Nylon foil was found to give better results than polythene foil in sterilization at low pressures. As a general rule, the articles to be sterilized should be wrapped in nylon before being placed in three-foil bags, since this considerably facilitates sterile handling when the bag

is opened. As mentioned, extraction of sterilized articles should be done either in the open air or under a closed hood so as to avoid inhalation of the escaping gas.

Bulky objects containing large volumes of air that cannot be manually forced out are not suitable for sterilization in three foil bags of the normal size since the trapped air will render the ethylene oxide concentration inadequate. For such articles gas sterilization should be preceded by a vacuum phase.

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Extension of the exposure results from these commercial sterilizers. The most rapid of all those tested gave satisfactory results.

The autoclave converted for ethylene oxide sterilization like the high pressure commercial sterilizers gave satisfactory results in tests using aluminium foil slips which had been moistened with a drop of water and were exposed in open dishes. In addition test slips wrapped in nylon foil together with a moist cottonwool swab were sterilized in all but one test. The preliminary vacuum phase and the longer exposure to ethylene oxide undoubtedly explain the superior results as compared with those from the high pressure commercial autoclave. The importance of the vacuum phase was also demonstrated by the experiments in which the test slip was placed in a cotton plugged test tube and then in a bag of polythene foil. With this arrangement the converted autoclave was clearly superior to the high pressure commercial autoclave. A pertinent point is that bags containing bulky articles should not be

completely sealed before sterilization, in order to avoid the danger of bursting of the foil during the vacuum phase. A small slit should be left open and can easily be sealed with tape immediately after sterilization.

Sterilized articles of rubber, plastic or other material in which ethylene oxide has become dissolved must be aired before use (Phillips & Kaye 1949, Grundy, Rdzok, Remo, Sagen & Sylvester 1957, Freeman & Barwell 1960). This is particularly important as regards articles which come into direct contact with respiratory passages, blood vessels or other tissues. (Ethylene oxide has also been successfully used for sterilizing various types of tissue grafts). In the converted autoclave a vacuum can be created also after sterilization, which considerably facilitates rapid removal of ethylene oxide. With the other methods airing at room temperature is necessary. The time of airing is dependent upon the density and nature of the sterilized articles.

As regards cost of ethylene oxide sterilization in the converted autoclave, the price of the "Cartox" gas is approximately 240 Sw. crowns per 25 kg, which implies that the cost of a run is about 30 crowns. In these experiments about 2 kg of gas were used per run. It is recommended that not more than four-fifths of the gas in the tube should be used, since the concentration of ethylene oxide in the last portion of the mixture may fall below the figure stated on the tube. The volume of the sterilization chamber is 500 liters and therefore large numbers of articles can be sterilized at one time.

## SUMMARY

Three methods for ethylene oxide sterilization are described.

- 1) In "three foil" bags,
- 2) in commercial sterilizers, and
- 3) in a converted steam autoclave.

1. The three-foil bag method can be used for ethylene oxide sterilization when special apparatus is not available. The foil is composed of paper, aluminium and plastic. It is impermeable to ethylene oxide and with the aid of an ordinary household iron, can easily be made into bags of desired size. The nonsterile material, which may be wrapped in nylon film, is placed in the three foil bag together with a small ethylene oxide from aerosol can, the valve of which can be manipulated from outside the bag. Instruments can preferably be dipped in water before being wrapped in plastic foils or can be placed directly in the three-foil bag, along with a source of moisture. If the articles have been stored under extremely dry conditions before sterilization, the direct moistening is essential. Exposure to ethylene oxide in three-foil bags for 6 hours at room temperature gives full sterility.

2. In a commercial ethylene oxide sterilizer which functions with a pressure of 6.4 kg per cm<sup>2</sup>, the same bacteriologic studies were made

as with the three foil bags. The results from the former method were less good undoubtedly because of the shorter period of exposure to the gas. Full sterility was nevertheless obtained when the test slips were treated openly in the sterilizing chamber and had previously been moistened with water. Wrapping in plastic of the articles to be sterilized is not recommended when this apparatus is used. The method is the quickest of those tested, the sterilizing time being one hour.

3. A 200 liter autoclave was modified so that it could be used for both heat and gas sterilization. When the temperature was  $60^{\circ}\text{C}$  and the chamber was filled with ethylene oxide-carbon dioxide up to a pressure of 2 atmospheres, full sterility was obtained after 6 hours of all test material in open dishes and in nylon bags provided that it had been directly moistened with water before sterilization. Even test material wrapped in nylon foil together with a moist swab was sterilized in practically all cases. This converted autoclave is particularly suitable when large numbers of articles or bulky objects are to be sterilized.

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- 2) in commercial sterilizers, and
- 3) in a converted steam autoclave.

1. The three-foil bag method can be used for ethylene oxide sterilization when special apparatus is not available. The foil is composed of paper, aluminium and plastic. It is impermeable to ethylene oxide and with the aid of an ordinary household iron, can easily be made into bags of desired size. The nonsterile material, which may be wrapped in nylon film, is placed in the three-foil bag together with a small ethylene oxide/aerosol can, the valve of which can be manipulated from outside the bag. Instruments can preferably be dipped in water before being wrapped in plastic foils, or can be placed directly in the three foil bag along with a source of moisture. If the articles have been stored under extremely dry conditions before sterilization, the direct moistening is essential. Exposure to ethylene oxide in three foil bags for 6 hours at room temperature gives full sterility.

2. In a commercial ethylene oxide sterilizer which functions with a pressure of 6.4 kg per cm<sup>2</sup>, the same bacteriologic studies were made

as with the three-foil bags. The results from the former method were less good, undoubtedly because of the shorter period of exposure to the gas. Full sterility was nevertheless obtained when the test slips were treated openly in the sterilizing chamber and had previously been moistened with water. Wrapping in plastic of the articles to be sterilized is not recommended when this apparatus is used. The method is the quickest of those tested, the sterilizing time being one hour.

3. A 500 liter autoclave was modified so that it could be used for both heat and gas sterilization. When the temperature was 60° C and the chamber was filled with ethylene oxide or carbon dioxide up to a pressure of 2 atmospheres, full sterility was obtained after 8 hours of all test material in open dishes and in nylon bags provided that it had been directly moistened with water before sterilization. Even test material wrapped in nylon foil together with a moist swab was sterilized in practically all cases. This converted autoclave is particularly suitable when large numbers of articles or bulky objects are to be sterilized.

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## IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS-REACTIONS IN THE KLEBSIELLA GROUP

### 9 Cross Reactions between *Klebsiella* Types 11, 21 and a Closely Related Strain

By

JORUN FRIKSEN and S D HANSEN

Received 22 xii 62

In a previous study (1) a *klebsiella* strain, W60, originally isolated from water, was found to give capsular reactions up to the homologous titre in immune sera against the sero types 11 and 21. The reactions obtained were the following

Strain	Immune serum	
	anti 11	anti 21
Type 11	16	16
Type 21	4	32
W60	32	32

After absorption of these two immune sera with the strain W60 the homologous antigens no longer showed a detectable capsular reaction. The results, therefore, suggested the possibility that the strain W60 possessed the type-specific antigens of both types and thus might be a "double" type similar to the artificial double types of *Haemophilus influenzae*, produced by *Leidy, Hahn & Alexander* (2), and of *Diplococcus pneumoniae*, produced by *Austrian et al* (3-6). This paper reports the results of a quantitative study of these cross-reactions.

### MATERIAL AND METHODS

The type strains of types 11 and 21 in the following designated strains 11 and 21 were originally received from *Dr F Kauffmann* Copenhagen. Strain W60 was isolated from a sample of drinking water. All strains had been kept in the lyophilic state for some years.

Preparation of immune sera and serological methods were as described previously (7).

Capsular polysaccharides were prepared from bacterial suspensions in water by a simplified method described in a previous paper (8). Qualitative chemical analysis was carried out by paper chromatography as described previously.

Agar diffusion precipitation analysis was carried out as described by *Ouchterlony* (10). Immune serum was put in a central well and antigens were distributed in 6 peripheral wells. In some instances the wells were refilled when empty.



## RESULTS

The composition of the capsular polysaccharides is shown in Table 1. The polysaccharides of strains 11 and W60 have the same composition whereas strain 21 lacks glucose, which is present in the two others.

The results of capsular reactions are shown in Table 2. The results confirm that the capsular polysaccharides of the three strains are closely similar serologically, but in these sera there are some differences between strain 21 and the two others.

The results of quantitative precipitin determinations are shown in Figs. 1 to 3. The nitrogen contents of the antigens used in these tests were 0.3 per cent (11, W60) and 0.2 per cent (21). The antigens 11 and W60 precipitate equal quantities of antibody from serum anti-11, whereas strain 21 precipitates only 42 per cent of the total antibody. From serum anti-21 the antigens 11 and W60 bring down about one half of the total precipitable antibody, W60 slightly more (57 per cent) than 11 (47 per cent). The difference is small, but suggestive when seen together with the results, obtained with the serum anti-W60. In this serum antigen 11 reacts only with 80 per cent of the antibody, and antigen 21 precipitates 32 per cent.

The results obtained with the gel diffusion method are illustrated in Figs. 4 to 6. Fig. 4 indicates that the serum 11 contains at least two different antibodies, one of which reacts with an antigen shared by strains 11 and W60, but apparently not present in antigen 21. The reactions of antigens 11 and W60 have the appearance of reactions of

TABLE 1  
*Chemical Composition of Capsular Polysaccharides Isolated from Klebsiella Strains 11, 21 and W60*

Strains	11	21	W60
Uronic acid	+	+	+
Glucose	+	0	+
Galactose	+	+	+
Mannose	+	+	+

TABLE 2  
*Capsular Reactions of Klebsiella Strains 11, 21 and W60 in Immune Sera against the same Strains*

Antigen	Immune serum		
	Anti 11	Anti 21	Anti W60
11	128*	16	64*
21	32	64*	32
W60	128	16	64*

Titres are given as the reciprocal of the highest serum dilution giving positive reaction.

\* Only weak reactions with the highest serum dilution.

identity, and with this arrangement of the wells there is no indication of any dissimilarity between these two antigens. The second antibody, which produces a much less distinct precipitate band, reacts with an antigen which appears to be present in all three strains, since an apparently complete ring of precipitate is formed around the central well.

The results obtained with serum anti-W60 (Fig 3) are less easy to explain, but suggest the presence of at least 2 antibody fractions. One of these reacts with an antigen common to strains 11 and W60, and produces a precipitate band which may correspond to the peripheral band in Fig 4. Like the latter, it appears to show a reaction of identity. The second band runs parallel to the first band except where it ap-

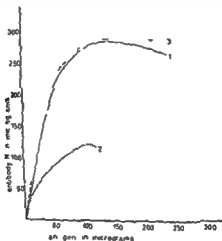


Fig 1

Quantitative precipitin determinations in serum anti 11 Curve 1 antigen 11 Curve 2 antigen 21 Curve 3 antigen W60

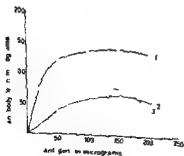


Fig 2

Quantitative precipitin determinations in serum anti 21 Curve 1 antigen 21 Curve 2 antigen 11 Curve 3 antigen W60

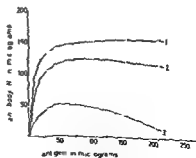
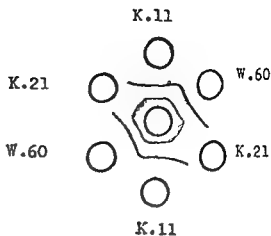


Fig 3

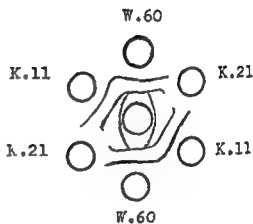
Quantitative precipitin determinations in serum anti W60 Curve 1 antigen W60 Curve 2 antigen 11 Curve 3 antigen 21

*Fig 4*

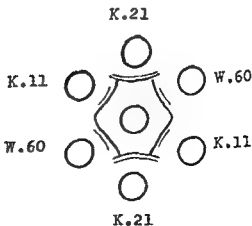
Gel precipitation test with serum anti 11 (central well) and the three antigens (peripheral wells)

*Fig 5*

Gel precipitation test with serum anti W60 (central well) and the three antigens (peripheral wells)

*Fig 6*

Gel precipitation test with serum anti 21 (central well) and the three antigens (peripheral wells)



proaches to the well containing antigen 21. Here the ends of the bands bend towards one another. At first sight, this might seem to suggest the presence of small quantities of the same antigen in strain 21, but since no visible precipitate is seen in the vicinity of the wells containing antigen 21, the effect is probably due to the advancing boundaries of antigen fractions from strains 11 and W60 only. More interiorly, towards the central well precipitate bands are seen opposite to the wells containing antigen 21. These bands fuse with a part of the bands just described, indicating a reaction of partial identity between an antigen contained in strain 21 and an antigen common to the two other strains. Thus the reaction of antigen 21 in this serum seems to be due to partial cross reactivity of a fraction of this antigen with one of the antigen fractions of the homologous strains.

Fig. 6 finally shows that antigen 21 contains two fractions, one of which shows a reaction of partial identity with an antigen common to strains 11 and W60.

There is a suggestion of a slight reaction between a second antigen fraction contained in strains 11 and W60 and this serum, but these precipitate bands were very faint, and the reactions may not be reliable.

#### DISCUSSION

The results confirm that the three strains show marked cross reactions in the immune sera against one another, but do not confirm that the strain W60 was a double type, as suggested by previous results. Two reasons for this discrepancy might be suggested. One is that capsular reactions carried out after absorption of the sera with cross-reacting bacterial antigens may be difficult to read due to decreased transparency of the absorbed sera. Thus remaining reactivity of the serum used in previous experiments may have been overlooked. The second is that the cross reactions obtained with immune sera from different rabbits may differ.

The two strains 11 and W60 appear to produce nearly identical capsular antigens. Indeed the results of gel precipitation tests suggest that the antigen precipitin determined by the reaction of the two strains. It is to an additional antigen in the preparation produced from strain W60, an antigen present in too small quantity to be detectable by gel precipitation. If so, these two strains may be considered to belong to the same capsular type. It also seems possible that the difference might be due to minor structural differences between the reactive groupings on one or the other pair of antigens. In case a reaction is observed in the studies are

The results show that the antigen preparation from each of the three

strains contains at least two distinct antigens. This is in agreement with results obtained in a study of the capsular antigen of *Klebsiella* sero-type 1 (11). It is not yet known whether this signifies that the capsule of such strains is composed of two polysaccharides, or that some other antigen, e.g. a somatic antigen has contaminated the capsular antigen. In order to decide this it will be necessary to separate the two antigens and to study their rôle in the capsular reactions of the strains.

These results indicate that the antigenic constitution of *Klebsiella* strains may be more complicated than expected.

### SUMMARY

Studies on the capsular antigens of three cross-reacting *Klebsiella* strains are reported. Two separate antigens could be detected in the antigen preparations from each of the three strains.

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## NON-PIGMENTED *SERRATIA MARCESCENS* VAR. *KIELENSIS* AS A PROBABLE CAUSE OF BRONCHOPNEUMONIA

By

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The normal habitat of *Serratia* is water and soil. While considered to be a strict saprophyte in the past, there is now growing evidence for its capability of producing human infection, especially in chronically debilitated patients (9, 10). *Serratia* has been found to be associated with skin and wound infections, infant diarrhea, infections of the urinary and respiratory tracts, sinusitis, otitis media, meningitis, endocarditis and septicæmia (7, 16, 20, 21 and other references cited). In some of the reported cases the pathogenetic rôle of *Serratia* may be questioned, but it seems wise to recognize this group of organisms as potential cause of serious infection, particularly since the advent of the widespread use of antibiotics capable of altering the bacterial flora of the human body, setting the stage for enhanced invasiveness and virulence on the part of these bacteria (2).

Next to the urinary tract the most frequently involved organ system in *Serratia* infections seems to be the respiratory tract.

We have found reports on only two cases of pneumonia considered to have been due to *Serratia*. Atloff *et al.* 1936 (1) reported pure culture of *ratia* from sputum in a case of pneumonia. Bernhard & Sutton 1962 (2) described a patient who became ill with *Serratia* pneumonia after 10 days of treatment with penicillin and tetracycline of a pelvic abscess caused by *Staphylococcus pyogenes* var. *aureus*.

Isolation of *Serratia* in chronic bronchitis has been described by Woodward & Clarke 1913 (22), Gale & Lord 1957 (8) and Robinson & Woolley 1957 (18). Gale & Sonnenwirth 1962 (9) reported mild respiratory infection possibly caused by *Serratia* in a case with previous lobectomy for bronchiectasis. Mild respiratory disease in laboratory workers exposed to aerosols of *Serratia marcescens*, has been reported by Paine 1946 (15) and by Reidman *et al.* 1955 (17).

The present publication is justified by the scarceness of previous reports on *Serratia* infections. In addition, the isolated *Serratia* variety is of interest because of its very rare identification in human materials.

and because there was need for an extended examination to clarify the bacteriological diagnosis

## CASE REPORT

TO 64 years old The patient had a severe influenza in 1918 Since then frequent attacks of upper respiratory disease have occurred involving cough expectoration and slight fever In 1955 he had an attack of thrombosis of the right carotid artery with left hemiparesis sequelae of which still persist His cough reflexes are weak

In the summer of 1961 he had bronchopneumonia and recovered after 14 days in bed In the middle of December 1961 he became ill again with fever, cough and yellow expectoration He was admitted to hospital on December 27, after having been treated with penicillin On admission his general condition was poor T<sub>p</sub> 38.7 Leucocyte count 26100 SR 90 mm Total serum proteins 5.4 g per 100 ml with reduction of albumin and elevation of  $\alpha_1$  and  $\alpha_2$  globulins Clinical and radiological examinations revealed left-sided lung infiltration and pleural effusion Diagnosis Bronchopneumonia

Penicillin and streptomycin treatment during the first days in hospital had little effect On January 1 1962 chloromycetin treatment was started The temperature then gradually returned to normal together with general and radiological improvement The leucocyte count decreased to 11300 on January 9 SR rose to 95 mm on January 14 days later it was 80 mm When the patient left hospital on January 20 1962 he was in a good condition One year later he is still healthy

## MATERIAL

**Bacteriological specimens** Expectations sampled on December 28 December 31 and January 5 were subjected to common bacteriological examination (Specimens sampled on December 31, January 1 and January 2 were cultured for tubercle bacteria with negative results)

**Blood specimens** Agglutination tests with patient sera were performed with blood sampled on January 5 15 and 20 1962

## METHODS

Primary cultures were made aerobically on blood agar plates and subcultures on

for the formation of acid from carbohydrates and in 1 per cent peptone water to which had been

added 1 per of paraffin procedure al

The absence of gas production in glucose and mannitol was controlled by means of Durham tubes

Reaction in d tartrate tests for nitrate reduction and indol production and methyl red and Voges Proskauer reactions were carried out according to Kauffmann (12) Utilization of citrate as the sole source of carbon was read in the conventional Koser's medium gelatin liquefaction in a medium of 15 per cent gelatin in broth and hydrogen sulfide production in TSI medium (Difco) The urease test was performed according to Hale Christensen (3) the KCN test according to Muller (14) glutamic acid decarboxylase activity test according to Shaw & Clarke (19) by their simplified technique and the phenylalanine reaction according to Henriksen & Gloss (11) by a slightly modified procedure (19)

Oxidase reaction was performed with a 1 per cent aqueous solution of dimethyl p phenylenediamine hydrochloride and with a 0.5 per cent freshly prepared aqueous solution of tetramethyl p phenylenediamine hydrochloride the catalase reaction with a 3 per cent hydrogen peroxide solution and ether sensitivity test in the following manner 1 volume of culture was shaken with 2 volumes of ether for 1 min and blood agar heavily inoculated from the read on coefficient serum slants and litmus skimmed milk

Flagellar staining was performed as described by *Leifson* with formaldehyde fixation (4), and antibiotic sensitivity tests according to the method of *Fricsson et al* (6).

*Serological procedure* An experimental antiserum was obtained by intravenous injection into a rabbit of 0.25, 0.5, 1 and 2 ml of a moderately turbid suspension of washed living bacteria from 6 hr old cultures on nutrient agar surfaces. Injections were undertaken at 5-7 days' intervals and bleeding 7 days after the last injection. This serum was compared with the 3 patient serum specimens in agglutination tests.

## RESULTS

*Cultural characteristics* All three primary cultures were pure and abundant. Single colonies on blood agar were roughly circular, measuring up to 3 mm in diameter after 24 hr at 37° C. They were convex and not swarming. As colonies aged, they showed a great tendency to form papillae. They were densely opaque and their consistency was butyrous. There was a slight smell of ammonia. Single colonies were non haemolytic, but heavier growth zones revealed pronounced haemodigestion. Growth on blood agar became brownish green when aged. On no occasion red pigment production was observed. On bromothymol blue lactose agar colonies were bluish green. After several days on this medium at room temperature a yellowish tinge of colonies was observed, while the medium was rendered more and more alkaline. Sectors or "tongues" of these old yellowish colonies were light blue in colour. In subculture these mutants showed no haemodigestion and were less active than the

reactions, except for lactose fermentation, which in the mutants could not be detected aerobically in 30 days (HCN test and glutamic acid decarboxylase test were not performed with the mutants).

Growth in liquid media was diffuse, without pellicle and with little sediment in young cultures.

Growth optimum was approximately 32-34° C, but abundant growth occurred at all temperatures between 28 and 37° C. Growth at 22° was willing, but less abundant. There was no growth at 41° C, and no survival after heating to 56° C for 30 minutes. The strain was a facultative anaerobe, but growth anaerobically was strongly depressed.

*Microscopical morphology* The strain was invariably Gram negative, not acid fast, and not sporulating. Some variation in

... intermingled by thin rods. Indian ink preparations made a narrow capsule evident. Motility in most ...

Serratia (13) ... shape, which has been described by *Leifson* in



TABLE 1  
Biochemical Behaviour

Adonitol	+	(3-4)	Raffinose	-	(30)
Dulcitol	-	(30)	II tartrate	-	(14)
Sorbitol	+	(1)	KNO <sub>3</sub> reduction	+	(1)
Arabinose	-	(30)	V P 1 day	-	
Xylose	-	(30)	V P 4 days	-	
Rhamnose	-	(30)	MR 1 day	+	
Maltose	+	(1)	MR 4 days	+	
Salicin	+	(1)	V P 2 days 22° C	-	
Inositol	+	(1)	MR 2 days 22° C	+	
Lactose	+	(1) (20)	Indol production	-	(4)
Sucrose	+	(1)	H <sub>2</sub> S production	-	(4)
Mannitol	+	(1)	Gelatin liquef	+	(1)
Glucose	+	(1)	Serum liquef	+	(1)
Trichalose	+	(1)	Citrate growth	+	(1)
Mannose	+	(1)	Urease production	-	(30)
Fructose	+	(1)	Phenylalanine reaction	-	
Galactose	+	(1)	hCN test	+	
Dextrin	+	(1)	Glutamic acid decarb	+	
Starch	+	(1)	Lithers sensitivity	+	
Glycogen	-	(30)	Catalase production	+	
Inulin	-	(30)	Oxidase reaction	-	
Glycerol	+	(1)	Litmus milk	Acid with clot in 1 day	
Cellobiose	-	(30)	Digestion of clot in 5-7 days		

In fermentation reactions + or - indicate acid or no detectable acid production aerobically. Added figures in brackets indicate time in days for reactions to appear or days of incubation in negative reactions. Incubation temperature 37° C if no other temperature is mentioned.

TABLE 2  
Tube Agglutination with three Patient Sera and one Rabbit Antiserum

Antigen	Serum			
	A	B	C	D
5 hr broth culture living bacteria	100	320	320	10240
5 hr broth culture heated to 60° C for 1 hr	0	0	0	640
20 hr agar culture suspension heated to 100° C for 2 hr	0	0	0	320
20 hr agar culture suspension heated to 120° C for 2 hr	0	0	0	320

Doubling dilution from 1 in 10 titres listed are inverse of last distinctly positive dilution. A, B, C are patient sera implect in January 5, 15 and 20, 1962 respectively. D is rabbit antiserum to living bacteria.

**Biochemical reaction.** See Table 1. The strain was invariably anaerobic in fermentation reactions also when tested at 22° C. When aerobic tubes turned acid there was also pronounced acid reaction anaerobically which made a fermentative mode of metabolism evident.

The strain was forwarded to Dr P. R. Edwards, Communicable Disease Center, Atlanta, Georgia. He reports that Dr W. H. Fwing considers it to be a typical strain of *Serratia marcescens* var. *kudzensis*.

**Serology.** Patient sera gave a moderate and somewhat slow agglutination in preliminary slide agglutination tests. In Table 2 results of tube agglutination with patient and rabbit antisera are listed.

**Pathogenicity to laboratory animals** White mice injected intraperitoneally with  $10^8$  living bacteria washed in saline died in 1 to 3 days. On the other hand the rabbit immunized with living bacteria intravenously did not show any sign of illness during one month.

**Chemotherapeutic sensitivity** The strain was highly sensitive to sulphonamide streptomycin and chloromycetin moderately sensitive to tetracyclines and resistant to penicillin kanamycin erythromycin and polymyxin B.

## DISCUSSION AND CONCLUSION

The identification of the Voges Proskauer negative *Serratia marcescens* var. *kielensis* is extremely rare in medical bacteriology (1). In the scarce literature on *Serratia* as possible human pathogen this variety has not been encountered. However as the lack of readily visualized pigment production is very frequent in *Serratia* (5, 7) it is probable that many cultures of *Serratia* have in the past been classified as *Aerobacter* or intermediate coliform bacteria (5). An exact estimate of the distribution and general importance in human pathology of *Serratia* including the *kielensis* variety is therefore impossible at present.

That our strain was found in abundant pure culture in three successive specimens from a case of clinical infection which responded well to the antibiotic treatment indicated in vitro are strong arguments for its pathogenicity and causal importance. Supporting evidence for this is also the observed antibody response. The stability of the antibody titre during 15 days may be explained by the probably longstanding illness. The titre was rather low but this may in part be explained by the generally poor condition of the patient. As a matter of fact lowered resistance may have been decisive for the infection.

The antigen reacting with patient sera did not withstand  $60^\circ\text{C}$  for 1 hr resembling the I antigen of *Escherichia* and the Vi antigen of *Salmonella* with regard to thermolability. A rabbit antiserum to living bacteria showed a similar peak of antibodies to a thermolabile antigen which showed the agglutinogenic capacity of the latter. The observed thermolability of this probably flagellar antigen may have connection with heat influence on the flagellar morphology.

## SUMMARY

Infant  
with

25-150  
bronchitis

choice according to in vitro tests. There was a specific antibody response in patient sera. It is concluded that the strain probably had an actual pathogenetic importance in the case reported.

TABLE 1  
*Biochemical Behaviour*

Adonitol	+	(3 4)	Raffinose	—	(30)
Dulcitol	—	(30)	D tartrate	—	(14)
Sorbitol	+	(1)	KNO <sub>3</sub> reduction	+	(1)
Arabinose	—	(30)	V P 1 day	—	
Xylose	—	(30)	V P 4 days	—	
Rhamnose	—	(30)	M R 1 day	+	
Maltose	+	(1)	M R 4 days	+	
Salicin	+	(1)	V P 2 days 22° C	—	
Inositol	+	(1)	M R 2 days 22° C	+	
Lactose	+	(16 20)	Indol production	—	(4)
Sucrose	+	(1)	H <sub>2</sub> S production	—	(4)
Mannitol	+	(1)	Gelatin liquef	+	(1)
Glucose	+	(1)	Serum liquef	+	(1)
Trichalose	+	(1)	Citrate growth	+	(1)
Mannose	+	(1)	Urease production	—	(30)
Fructose	+	(1)	Phenylalanine reaction	—	
Galactose	+	(1)	hCN test	+	
Dextrin	+	(1)	Glutamic acid decarb	+	
Starch	+	(1)	Other sensitivity	+	
Glycogen	—	(30)	Catalase production	+	
Inulin	—	(30)	Oxidase reaction	—	
Glycerol	+	(1)	Litmus milk	Acid with clot in 1 day	
Cellobiose	—	(30)	Digestion of clot in 5 7 days		

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Doubling dilutions from 1 in 10. Titres listed are inverse. Last distinctly positive dilution. A C are patient sera sampled on January 5 15 and 20 1962 respectively. D is rabbit antiserum to living bacteria.

**Biochemical reaction** See Table 1. The strain was invariably anaerogenic in fermentation reactions, also when tested at 22° C. When aerobic tubes turned acid, there was also pronounced acid reaction anaerobically, which made a fermentative mode of metabolism evident.

The strain was forwarded to Dr P R Edwards, Communicable Disease Center, Atlanta, Georgia. He reports that Dr W H Lwing considers it to be a typical strain of *Serratia marcescens* var. *kielensis*.

**Serology** Patient sera gave a moderate and somewhat slow agglutination in preliminary slide agglutination tests. In Table 2 results of tube agglutination with patient and rabbit antisera are listed.

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**Chemotherapeutic sensitivity** The strain was highly sensitive to sulfonamide, streptomycin and chloromycetin, moderately sensitive to tetracyclines and resistant to penicillin, kanamycin, erythromycin and polymyxin B.

## DISCUSSION AND CONCLUSIONS

The identification of the Voges-Proskauer negative *Serratia marcescens* var. *kienensis* is extremely rare in medical bacteriology (3). In the scarce literature on *Serratia* as possible human pathogen, this variety has not been encountered. However, as the lack of readily visualized pigment production is very frequent in *Serratia* (5, 7), it is probable that many cultures of *Serratia* have in the past been classified as *Aerobacter* or intermediate coliform bacteria (3). An exact estimate of the distribution and general importance in human pathology of *Serratia*, including the *kienensis* variety, is therefore impossible at present.

That our strain was found in abundant pure culture in three successive specimens from a case of clinical infection which responded well to the antibiotic treatment indicated *in vitro* are strong arguments for its pathogenicity and causal importance. Supporting evidence for this is also the observed antibody response. The stability of the antibody titre during 15 days may be explained by the probably longstanding illness. The titre was rather low, but this may in part be explained by the generally poor condition of the patient. As a matter of fact, lowered resistance may have been decisive for the infection.

The antigen reacting with patient sera did not withstand  $80^\circ\text{C}$  for 1 hr, resembling the L antigen of *Escherichia* and the Vi antigen of *Salmonella* with regard to thermolability. A rabbit antiserum to living bacteria showed a similar peak of antibodies to a thermolabile antigen which showed the agglutinogenic capacity of the latter. The observed thermolability of this probably flagellar antigen may have connection with heat influence on the flagellar morphology.

## SUMMARY

A non-pigmented strain of *Serratia marcescens* var. *kienensis* was isolated repeatedly in pure culture from expectorations in a case of broncho-pneumonia which responded well to the antibiotic treatment of choice according to *in vitro* tests. There was a specific antibody response in patient sera. It is concluded that the strain probably had an actual pathogenic importance in the case reported.

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## IMMUNOELECTROPHORETIC ANALYSIS OF BLOOD STAINS WITH SPECIAL REFERENCE TO Gc GROUPING

By

H. VESTROM and J. SHAFTE JENSEN

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Grouping of blood stains, which is of great importance within forensic medicine, has in practice until recently been possible with regard to the ABO system only.

Adoption of new principles such as the mixed agglutination (1) and adsorption elution principle (5) has not only enabled ABO grouping of very small stains, but also allowed demonstration of the M, N-, and D-factors in stains (7). However, elaboration as to the number of applicable systems would be of significant value.

Reports on the demonstration of hapto-globin groups in blood stains (2) as well as preliminary results of Gm grouping of blood stains in this laboratory (3) seem to indicate that the genetically determined polymorphism of the serum proteins may become an important part of the analysis of blood stains.

This work presents the results of some preliminary immunoelectrophoretic examinations of blood stains with a view to Gc grouping.

### MATERIAL AND METHODS

#### *Blood Stains on Fabrics*

Examinations were made of a number of stains on linen stained with whole blood of 3 donors of types Gc 1-1, Gc 2-1 and Gc 2-2 respectively. The stains originating from the Gc 1-1 donor were examined after 2 days of storage, the stains from the Gc 2-1 donor after 3 days and the stains from the Gc 2-2 donor after 2, 6 and 7 days. From the Gc 1-1 donor further stains, consisting partly of whole blood, partly citrated blood, were made on linen and silk and examined when 3 hours old.

All stains were allowed to dry and were stored at room temperature.

#### *Blood Stains on Filter Paper*

Of 4 sets of filter paper strips 2 × 2 mm (Whatman no. 3) one was moistened with 3 microliters of blood from the above donor of type

Gc 1-1, another from the donor of type Gc 2-2 and the remaining two with blood from 2 new donors of type Gc 2-1. The stains were left to dry and stored at room temperature. With a view to a more systematic study of the storage period's influence on Gc-grouping, the examination of stains from the 4 sets was performed at increasing intervals, from after 4 hours and up to 15 days of storage.

### *Serum Stains on Filter Paper*

Finally, sets of filter paper strips  $2 \times 2$  mm were moistened with  $1\frac{1}{2}$  microliter serum from 2 donors of type Gc 2-1 and 2-2. The strips were stored at room temperature and examined at increasing intervals after storage from 1 to 55 days.

### *Immunoelectrophoresis*

The employed technique is described in detail in an earlier publication (6). The adaptation of the technique to examination of the serum proteins in blood- and serum stains required few modifications only.

The blood- and serum stains on filter paper were examined by insertion of the filter paper strips into the agar at the site of the antigen basin.

In some cases blood stains on fabric were examined in the same way by insertion of a  $2 \times 2$  mm piece of the fabric into the agar. In other cases a stain eluate was made and applied to the antigen basin in a quantity of 1 to 2 microliters.

For the elution saline 0.9 per cent as well as distilled water were used and the process was carried out at room temperature over a period of from 1 to 24 hours. In the first examinations the quantity of the solvent was calculated on the basis of the dry matter weight within the particular stain under examination in an attempt to maintain the same dry matter content in the eluate as in blood (about 10 per cent). However, in later experiments stains of  $1 \text{ cm}^2$  were soaked with as small a quantity of solvent as possible and the eluate was squeezed out of the material.

In a few cases a further concentration was attained by evaporation of the eluate onto a  $2 \times 2$  mm filter paper strip used for the insertion into the agar.

At the immunoelectrophoretic examination the stain material was placed opposite a serum reference of a known Gc type.

The distance between the antigen basin and the antiserum trough was usually 5 mm but experiments have been carried out with distances as short as 1 mm.

The presence of small amounts of Gc substance in a stain eluate was demonstrated by an absorption test performed on an agar slide furnished with a centrally placed antigen basin containing reference serum and two lateral troughs, one holding immune serum, the other equal parts of immune serum and stain eluate.

Three different antisera were used (R 146, R 214 and R 225). Each antiserum was produced at the laboratory by injection of human serum into rabbits.

The results were read from the native as well as from the amido-black stained preparations. Each native preparation was photographed according to the so-called indirect method.

## RESULTS

*Blood Stains on Fabrics*

The analysis of blood stains on different fabrics displayed immunoelectrophoretic patterns, which for the majority of the precipitates did not deviate from normal serum patterns. In fresh stains the symmetry with the serum reference was complete, whereas in older stains a certain decrease was noted in the number of arcs as well as a cathodic extension of some arcs, most pronounced in the case of the albumin arc.



Fig 1

Immunoelectrophoretic pattern of a blood stain on silk stored for 3 hours and originating from a donor of type Gc 1-1 (above) Serum from the same donor (below) Arrows indicate the Gc the new alpha 1 globulin and the reaction of immunological identity Antiserum R 146

These trends, however, did not hold true for the Gc precipitates. In 3 hour old stains from a donor of type Gc 1-1 a distinct Gc 1-1 precipitate was found, but the distance between the precipitate and the immune serum trough was increased and the precipitate showed further characteristics of reduced concentration. Co-existing, in the alpha-1 globulin region, a precipitate occurred with which the Gc precipitate formed an identity reaction (Fig 1). In 2 day old stains from the same donor a weak Gc 1-1 precipitate was demonstrable using stain eluate for the application whereas no normal Gc precipitate was displayed by direct insertion of the stain material into the gel. In both cases the new arc occurred in the alpha 1 globulin region.

In 2 day-old stains from a donor of type Gc 2-2 a questionable Gc 2-2 precipitate was found at a considerable distance from the immune serum trough and a new arc similar to the one described above appeared in the alpha-1 globulin region.

In 3-day-old stains (type Gc 2-1) as well as in 6 and 7 day-old stains (type Gc 2-2) no Gc precipitate could be demonstrated, but the new arc in the alpha-1 globulin region was observed. Through absorption with an eluate from the 3 day-old stain of type Gc 2-1 anti Gc was removed from the immune serum.



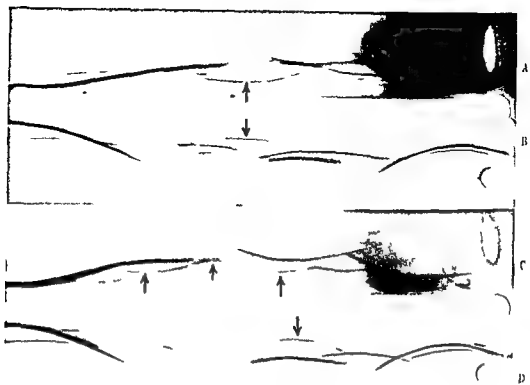


Fig 2

Immunoelectrophoretic pattern of blood stains on filter paper stored for 13 hours and originating from donors of type Gc 1 1 (A) and Gc 2 2 (C) opposite corresponding sera (B and D) Antiserum R 146

### Blood Stains on Filter Paper

Examination of blood stains on filter paper was performed at brief intervals beginning with hours' and increasing to days' intervals between tests. The Gc precipitates were found to be weakened in stains

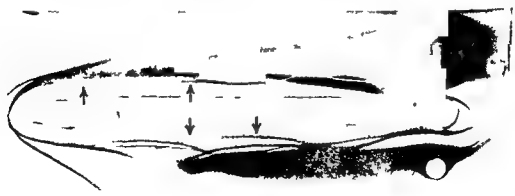


Fig 3

Immunoelectrophoretic pattern of a blood stain on filter paper stored for 48 hours and originating from a donor of type Gc 2 1 (above) Serum from the same donor (below) Antiserum R 214



Fig 4

Immunoelectrophoretic pattern illustrating the establishment of the immunological identity reaction between a weak Gc 2 component and the "new alpha 1 globulin" by admixture of serum of type Gc 1-1 to a blood stain on filter paper stored 48 hours and originating from a donor of type Gc 2-2 (above). Serum reference of type Gc 2-1 (below). Antiserum R 146.

less than 24 hours old. Reliable Gc grouping was only possible within the first approximately 2 days and then only of Gc 1-1 and Gc 2-2 stains (Fig 2), the Gc 2-1 precipitates appearing non-characteristic, asymmetric with a severely weakened 2 component (Fig 3).

In a few older stains of type Gc 1-1 the Gc type was recognizable.

In nearly all cases coexisting with the weakening of the Gc precipitates the new arc is observed by the examination of the stains on fabrics occurred in the alpha 1 globulin region. The immunological reaction between the Gc precipitate and the new arc was most distinct in the stains of type Gc 1-1 and 2-1. In the stains of type Gc 2-2 the

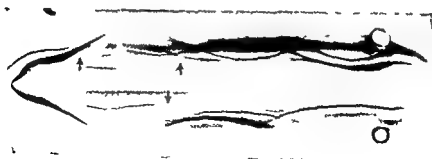


Fig 5

Immune  
Above  
globulin  
by qu

identity reaction was sometimes weak, but could be established by adding serum of type Gc 1-1 to the stain (Fig 4)

### *Serum Stains on Filter Paper*

In serum stains the Gc substance was much more stable. Flattening and blurring of the Gc precipitates did not occur until after about 1 month's storage, i.e. in time with the other arcs in the preparation. The deterioration was particularly pronounced in the case of the Gc 2-1 precipitates. The experiment was extended to 2 month old stains, in which weak and non-characteristic Gc precipitates appeared.

The above mentioned precipitate in the alpha-1 globulin region, occurring in the blood stains, was not observed in serum stains on filter paper.

### DISCUSSION

A total of more than 100 individual examinations of blood stains on fabric were performed, but owing to the variety of fabrics, methods of application etc. the number of uniform stains examined with the same technique is too small for a detailed analysis of the results.

However, it can be established that the Gc 1-1 and 2-2 types were demonstrable in blood stains a few days old. Further that a new precipitate in the alpha-1 globulin region showing immunological identity to Gc appears as the normal Gc precipitates are weakened and disappear. The ability of the stain eluate to absorb anti Gc was shown to be preserved in spite of the disappearance of the normal Gc precipitates. Investigations of blood stains on filter paper have yielded results largely corresponding with these findings.

Gc 1-1 precipitates have been demonstrable after storage of longer duration than Gc 2-2 precipitates, while Gc 2-1 precipitates have been found asymmetric showing a relative weak 2-component. Whether these findings are due to coincidence, are a characteristic feature of the samples employed, or are due to a weaker resistance on the part of the 2 component towards the effect of storage, can only be decided through more extensive examinations.

In contradistinction to the results of the examination of blood stains it has been possible to determine the Gc group of serum stains of approximately the same serum protein content as the blood stains after storage for several weeks. The present investigation does not offer any explanation of this difference between blood- and serum stains.

With regard to the new alpha-1 globulin component immunologically identical with Gc it should be emphasized that its electrophoretical position was independent of the Gc type of the stain. Further it may be mentioned that the precipitate was observed with the use of different antisera having in common a powerful anti Gc. The precipitate was only demonstrated in blood stains not in serum stains. In this connec-

tion, however, it should be mentioned that a similar precipitate has been observed in a few sera among a number of poor quality samples from Greenland even though the serum has been separated from the blood cells in Greenland. An example is given in figure 5. The immunoelectrophoretic pattern of so called pathologic serum as shown by Hirschfeld (5) manifests a precipitate with a similar position.

It appears that the Gc substance during unfavourable storage and under the influence of unknown factors undergoes a gradual transformation into a substance with an electrophoretical mobility like that of an alpha 1 globulin without losing its immunological specificity. It is found that this transformation takes place rapidly in dried blood stains, but nothing is known about the nature of this so called transformation. A partial decomposition or binding to decomposition products in the serum can be mentioned as possibilities.

### SUMMARY

Immunoelectrophoretic examination has been performed of a limited number of blood stains on different fabrics as well as of blood and serum stains on filter paper. The Gc groups of serum stains were demonstrable after storage at room temperature for 1 to 2 months. The Gc substances in blood stains were found to be very labile, the Gc precipitates presenting a rapid weakening and disfiguration. The appearance of a precipitate in the alpha 1 globulin region immunologically identical with the Gc precipitates was demonstrated. The results are discussed and it is concluded that the Gc under unfavourable storage and particularly in blood stains may undergo transformation into a faster migrating but immunologically identical component.

The extent of the present study does not allow any evaluation of the

applicability to completely fresh stains

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## ON THE PRESENCE OF GROUP-SPECIFIC G<sub>m</sub>- AND G<sub>c</sub>-SUBSTANCES IN URINE

By

JØRGEN C. NIELSEN, BENNY NIELSTRØM and Mogens FEJHO

Received 11 vii 62

Serum grouping according to the genetically determined G<sub>c</sub>- and G<sub>m</sub>-systems is based upon serologically and immuno-electrophoretically demonstrable polymorphism within the alfa-2- and gamma globulin fractions of the serum proteins.

As certain biological fluids contain serum proteins, though in varying quantities, serum grouping according to the systems in question might be possible. As regards the G<sub>c</sub>-system the group-specific substance has been demonstrated in certain samples of cerebrospinal fluid, in ascitic fluid and extravasates (9). Concerning the G<sub>m</sub>-system similar reports are not available, but in a few samples of synovia from knee joints one of us (J. C. N.) has been able to demonstrate the G<sub>m</sub> type of the patients.

As even normal urine contains traces of serum proteins (11) including the alfa-2 and gamma globulin fractions (16, 2, 15), it might be possible to demonstrate G<sub>c</sub> and G<sub>m</sub> substances in urine. Hirschfeld's (9) suggestion of the presence of G<sub>c</sub> substance in urine agrees with this assumption.

The present paper is based upon serological and immuno-electrophoretic examination of a small number of concentrated normal and proteinuric urine samples with a view to the occurrence of group-specific G<sub>m</sub> and G<sub>c</sub> substances in urine.

### MATERIALS AND METHODS

Urine. 1) Samples of normal human urine were supplied by healthy adults (members of the laboratory staff). 2) Samples of proteinuric urine were furnished by pregnant women from the Maternity Ward, Department B, Rigshospitalet, Copenhagen during the experimental period. The only criterion applied to the selection of the patients was the presence of proteinuria demonstrable by Heller's test. The degree of proteinuria as estimated by the Esbach test varied from 0.3-10 per thousand. The urine which was passed under simple precautions to avoid contamination was collected during a period of 12-24 hours. The cause of proteinuria in all cases was preeclampsia from mild to severe degrees, none of the cases

presenting a basis for the assumption that any chronic diseases of the heart or the kidneys were manifest. The age of the patients ranged from 17 to 37 years. Six of the patients were I parae two II parae one IV para and one V para.

*Process of concentration* The urine was concentrated by dialysis. The volumes of urine employed for concentration in the first experiments were 40-50 ml. In later

merthiolate was added both to saline and dextran solutions. The degree of concen

not taking into consideration the non protein nitrogen. Determination of the fractions of serum proteins were based upon paper electrophoresis. Total serum protein was determined by the Biuret test.

### Cm Group Determination

The principle of the group determination is a specific inhibition of different

*Imm Technique* Of the urine concentrate series of doubling dilutions 1:1:1:1:1 were made. The

Agglutination was graded as + + + + + Complete inhibition of the agglutination was designated —

### Ge Group Determination

The

immunospecificity was determined by electrophoretic trough between the two basins upon which diffusion took place for 24 hours at 37° C.

The immune serum concerned a rabbit anti-human serum.

Verification test as to the identity of the Ge precipitates of the urine was performed according to the principles described by Hirschfeld (10). Production of identity reaction between Ge of urine proteins and Ge of serum proteins was attempted by variation in the length of the immune serum trough as well as by production of a horse shoe shaped trough around 2 antigen basins one charged with urine concentrate the other with serum.

Thanks are due to Poul Astrup MD Rigshospitalet Copenhagen who kindly performed the Kjeldahl nitrogen analyses.

## RESULTS

The samples of normal urine were concentrated approximately 100 times as estimated by control weighings, but protein concentrations detectable by Kjeldahl's nitrogen analysis were not obtained. The samples of proteinuric urine were concentrated 5-50 times. This marked variation was due to differences in the duration of the dialysis against dextran as well as differences in the protein contents of the samples prior to concentration. The final protein contents of the proteinuric urine samples ranged from 1.1-12.1 per cent. This variation in the protein contents made it possible to estimate the sensitivity of the technique in the subsequent Gm and Ge grouping. Protein concentrations approximately equal to those of serum were obtained in only 4 urine concentrates.

TABLE 1

*Gm and Ge Group Determination of Concentrates of Proteinuric Urine Samples and the Corresponding Sera*

Journal No	Serum		Urine Concentrates		
	Gm	Ge	Protein Content per cent	Gm	Ge
B 106/62	a+b+γ-	1-1	1.1		
B 1944/61	a-b+γ-		1.5		
B 2369/61	a+b+γ-	2-1	1.6		
L 1011/61	a+b+γ-	1-1	3.1		
B 2254/61	a+b+γ-	2.1	abt 4		
B 2261/61	a+b-γ+	2.1	abt 4		
B 132/62	a+b+γ-	1.1	6.9	a+b+γ-	1-1
B 181/62	a-b+γ-	2.1	9.2	a-b+γ-	2.1
B 102/62	a+b+γ+	1.1	10.0	a+b+γ+	1.1
B 2041/61	a+b+γ-	2.1	12.1	a+b+γ-	2.1

The total serum protein values in the donors of the proteinuric urines ranged from 4.7 to 6.9 per cent. The fractions of serum protein did not differ from what would be expected in cases of pre-eclampsia, i.e. albumin and gamma globulin values moderately decreased, alpha- and beta-globulin values proportionally raised.

### *Results of the Gm Grouping*

The few samples of concentrated normal urine did not contain any demonstrable Gm substance. The concentrates of proteinuric urines were less inhibiting than the corresponding sera, but they appeared to allow group determination in 4 cases in which the protein contents ranged from 6.9-12.1 per cent. An example is shown in Table 2. In the remaining 6 cases group determination was not possible, the degree of inhibition for one or more of the Gm factors being too small to be conclusive.

TABLE 2  
Gm Group Determination of Urine Concentrate and Serum Originating in Patient  
no B 132/62 (Gm Group Gm(a+b+x-))

Agglutinins	Samples to be examined	Dilution of samples					
		1:1	1:2	1:4	1:8	1:16	1:32
Gm(a)							
Serum '1604' 1:20 anti D "07"	B 132/62 Serum Controls	—	— — — +	— — — ++ +	— — — ++ +	— — — ++ +	— — — ++ +
	Urine conc Serum Gm(a+) Gm(a-)						
							++ ++ ++ ++ ++ ++ ++ ++
Gm(b)							
Serum 'Berg' 1:16 anti D "42"	B 132/62 Serum Controls	—	— — — +	— — — ++ +	— — — ++ +	— — — ++ +	— — — ++ +
	Urine conc Serum Gm(b+) Gm(b-)						++ ++ ++ ++ ++ ++ ++ ++
Gm(x)							
Serum "Rolf" 1:10 anti D "07"	B 132/62 Serum Controls	++	++ ++ — +	++ ++ ++ ++ ++	++ ++ ++ ++ ++	++ ++ ++ ++ ++	++ ++ ++ ++ ++ ++ ++ ++
	Urine conc Serum Gm(x+) Gm(x-)						



## RESULTS

The samples of normal urine were concentrated approximately 100 times as estimated by control weighings, but protein concentrations detectable by Kjeldahl's nitrogen analysis were not obtained. The samples of proteinuric urine were concentrated 5-50 times. This marked variation was due to differences in the duration of the dialysis against dextran as well as differences in the protein contents of the samples prior to concentration. The final protein contents of the proteinuric urine samples ranged from 1.1-12.1 per cent. This variation in the protein contents made it possible to estimate the sensitivity of the technique in the subsequent Gm and Gc grouping. Protein concentrations approximately equal to those of serum were obtained in only 4 urine concentrates.

TABLE 1

*Gm and Gc Group Determination of Concentrates of Proteinuric Urine Samples and the Corresponding Sera*

Journal No	Serum		Urine Concentrates		
	Gm	Gc	Protein Content per cent	Gm	Gc
B 106/62	a+b+x-	1-1	1.1		
B 1934/61	a-b+x-		1.5		
B 2369/61	a+b+x-	2.1	1.6		
L 1011/61	a+b+x-	1.1	3.1		
B 2254/61	a+b+x-	2.1	abt 4		
B 2261/61	a+b-x+	2-1	abt 4		
B 132/62	a+b+x-	1-1	6.9	a+b+x-	1.1
B 181/62	a-b+x-	2-1	9.2	a-b+x-	2-1
B 102/62	a+b+x+	1-1	10.0	a+b+x+	1.1
B 2041/61	a+b+x-	2.1	12.1	a+b+x-	2-1

The total serum protein values in the donors of the proteinuric urines ranged from 4.7 to 11.9 per cent. The fractions of serum protein did not differ from what would be expected in cases of pre-eclampsia, i.e. albumin and gamma globulin values moderately decreased, alpha- and beta-globulin values proportionally raised.

### *Results of the Gm Grouping*

The few samples of concentrated normal urine did not contain any demonstrable Gm substance. The concentrates of proteinuric urines were less inhibiting than the corresponding sera, but they appeared to allow group determination in 4 cases in which the protein contents ranged from 6.9-12.1 per cent. An example is shown in Table 2. In the remaining 6 cases group determination was not possible, the degree of inhibition for one or more of the Gm factors being too small to be conclusive.

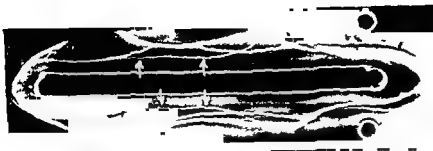


Fig 2

Immunoelectrophoretic pattern illustrating the verification of the identity of a slightly displaced Cc 1-1 precipitate originating in a urine concentrate (B 132/62) by admixture of an equal volume of serum reference of type Cc 2-2 (above)  
 Serum reference of type Cc 2-1 (below)      Antiserum B 146

poorly stored serum samples, viz a drawing out of the precipitates in the direction of the anode and weakening of the 2-component for instance in samples of Gc type 2-1. In all 3 cases the group determination was verified by repeated analyses after admixture of sera of known Gc types and by absorption tests. An example is given in Fig 2.

In 4 cases distinct patterns appeared, but individually without any unquestionable Gc arc or with a Gc-like precipitate, the identity of which, however, could not be verified. In one of these concentrates the absorption test indicated the presence of Gc substance.

In the last 2 cases few precipitates were seen, one of which the albumin arc

The employed immune serum did not allow a complete analysis of the protein fractions of the urine concentrates. The patterns however displayed in every case distinct albumin and transferrin precipitates. Furthermore precipitates appeared in the alpha 1 and alpha 2 region, the latter of a certain resemblance to hp and macroglobulin as shown in Figure 3. The presented observations on precipitates mentioned by has not been to urinary sediments in the antigen basin followed by a successive liberation of the protein.

The results of the Gm and Gc group determination of the urine concentrates appear from Table 1.

## DISCUSSION

The present investigation has demonstrated the presence of group specific Gm and Gc substances in concentrated urine samples from pregnant women suffering from preeclampsia, provided the protein contents of the urine concentrates were equal to or higher than 6.9 per cent. This finding is concordant with the observation made by others (15-7-18) that proteinuric urine, including urine from patients suf-



Fig 1

Immunoelectrophoretic pattern of a urine concentrate originating in donor B 102/62 showing a typical Ge 1 1 precipitate (A). Admixture of serum of type Ge 1-1 to the urine concentrate does not change the Ge precipitate (C) whereas admixture of serum of type Ge 2 2 produces a Ge 2 1 precipitate (E).

- B Serum from the donor B 102/62
- D Serum reference of type Ge 1 1
- F Serum reference of type Ge 2 2
- Antiserum used throughout R 146

### Results of the Ge Grouping

Ge grouping of concentrated normal urine was not possible, but normal urine in a single case was able to remove anti-Ge from immune serum by absorption.

The immuno-electrophoretic patterns of the concentrates of proteinuric urines allowed Ge grouping in the same 4 cases in which Gm grouping was possible. In one of these cases the appearance was very much like that of a normal serum pattern (Fig 1). The Ge arc was indistinguishable from a normal Ge immuno-precipitate of serum belonging to Ge type 1-1. Admixture of serum belonging to this type did not modify the Ge arc of the urine concentrate, whereas the addition of serum belonging to Ge 2 2 produced a clearcut identity reaction. In the absorption test all anti-Ge was removed. In the other 3 cases the immuno-electrophoretic patterns were slightly altered, as it may be seen in older,

ployed technique, nor may contamination with urine be assumed to interfere with Gm and Gc grouping of blood stains

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fering from the nephrotic syndrome, contains several serum proteins though in varying concentration, including alfa-2-globulins and gamma globulin

As regards the few examined concentrated normal urine samples Gm and Gc group determination was not possible. Only in a single case did the absorption test reveal the presence of Gc substance, but in a concentration that did not allow Gc grouping.

A probable explanation of this seems to be insufficiency of the employed process of concentration. The techniques used for Gm and Gc determination in the present work appear to require a protein concentration of the examined sample approximating that of serum. Qualitative and quantitative differences in the proteins of normal and pathological urines may play a certain rôle, but it must be stressed that normal as well as pathological urines contain serum protein fractions including alfa-2 and gamma-globulins. (For a comprehensive survey see 18)

From a theoretical point of view the importance for forensic medicine of the presence of Gm and Gc substances in urine seems obvious. The extent of the present investigation does not allow any estimation of the practical consequences of this observation. However, the results seem to suggest that Gm and Gc grouping of urine stains from normal or even from proteinuric urines will not be possible, on the other hand contamination with urine cannot be assumed to interfere with Gm and Gc grouping of blood stains (14, 13)

### SUMMARY

A series of normal and proteinuric human urine samples were examined with regard to the Gm and Gc contents.

The samples were concentrated by dialysis against a 10 per cent dextran solution. In the Gm group determination, comprising the factors Gm(a), Gm(b) and Gm(x), a titration technique was employed. The Gc group determination was performed by immuno electrophoresis.

The concentration technique proved inadequate for normal urine. The proteinuric urine samples were concentrated to a final protein concentration of 1.1 to 12.1 per cent. Gm and Gc group determination was practicable in 4 cases, in which the protein contents of the concentrates ranged from 1.9 to 12.1 per cent. No discrepancies between the Gm and Gc types in urine and the corresponding sera were found. In the remaining 6 cases, in which Gm and Gc grouping was not practicable, the protein contents of the concentrates were under 5 per cent.

The results are compared with previous immuno electrophoretic examinations of urine proteins. The extent of the present investigation does not allow any estimation of the consequences for medico-legal practice of the findings. However, the results seem to suggest that Gm and Gc grouping of stains of even proteinuric will not be possible by the em-

# TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

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Received ■ in 63

## 1 *M. Vastell* THE GENERAL APPEARANCE OF THE BRONCHIAL MUCOSA IN BRONCHIAL CANCER

The bronchial mucosa has been investigated in 50 cases of bronchial carcinoma and 56 control cases. Basal cell hyperplasia was often found in both series. A similar alteration ("pretransitional epithelium") and also "slit formation and expulsion" were however more common in the cancer material. A slit is formed underneath the surface cells resulting in a low epithelium without cilia. This probably provides the histopathological explanation for Papanicolaous Ciliocytophthoria. Keratinized squamous epithelium was found in carcinoma cases only and always within the tumour. Papilloma like stromaproliferations ("micropapilloma") had occurred in 60 and 25 per cent respectively of the cases while metaplastic epithelium (formed via basal cell hyperplasia or low epithelium) was found in 80 and 34 per cent respectively. Advanced epithelial atypia and extensive metaplasia was observed only in the cancer series and the former only in connection with transitional metaplasia. This suggested the existence of a generalized epithelial disturbance in the cancer cases.

## 2 *Th. Berge and Ö. Grönroft* CYTOLOGICAL DIAGNOSIS OF PLEURAL MESOTHELIOMA

## 3 *O. Grönroft* HISTOPATHOLOGICAL DIAGNOSIS OF NEEDLE BIOPSIES FROM THE PROSTATE GLAND (prel. communication)

## 4 *A. Bäckgren, L. E. Johansson, A. Norden, S. Rudarh and G. Wenqvist* SPONTANEOUS LEUCOSIS IN DOGS

In Sweden leucosis in dogs is not uncommon. For the last 25 years this disease has been found in 2 per cent of the autopsy material in the Department of Pathology of the Royal Veterinary College. The 200 cases of leucosis comprised 179 cases of a lymphatic type, 17 of a myeloid type and 3 of a plasmacell type. The disease was found in dogs 4-13 years of age. The mean age was 6.7 years. The mean age for the whole autopsy material during this period was 4.32 years. Sex distribution of the whole material was 51.5 per cent males and 48.5 per cent females. In the leucosis material 58.8 per cent were males and 41.2 per cent females. The disease seems to be predominant in males, this is however not significant. As carcinoma in dogs leucosis appears most frequently in boxers and in Airedale terriers. In connection with the advanced mean age this perhaps may be an expression for the blastomatous character of leucosis.

## BRIEF REPORT

### QUANTITATIVE DATA ON THE CELL CONTENT OF THE FEMORAL BONE MARROW IN THE ADULT FEMALE ALBINO RAT

By F. Biering and I. Grunnet

The technique adopted in the present investigations differs in only slight detail from that described by Ramsell & Ioffey (1961) for quantitative examination of the bone marrow in the rat.

Under ether anaesthesia the animals were killed by bleeding through incision of the abdominal aorta. The blood was collected, centrifuged and the serum pipetted off. Both femurs were removed immediately after the bleeding. They were cleaned, the epiphyses sawn off and the diaphyses immediately carefully split lengthwise. The bone marrow cylinders thus exposed were transferred to a glass tube with a tight fitting rubber stopper containing a previously weighed amount of the autogenous serum sufficient to give a dilution of bone marrow in serum of approximately 1:15. The bone marrow cells were suspended in the serum by vigorous mechanical shaking for three minutes. The suspension was weighed and the specific gravity of the suspension and the serum was determined by the copper sulphate method. The volume of suspension and of serum was calculated and from these values the volume of bone marrow used was determined. The volume dilution of bone marrow in serum could then be calculated. The absolute number of nucleated cells per  $\text{mm}^3$  of suspension—counted in a haemocytometer after dilution (1:40) with a solution of methyl violet and acetic acid—was converted into the number per  $\text{mm}^3$  of bone marrow by means of the volume dilution. The absolute number of erythrocytes per  $\text{mm}^3$  of bone marrow was determined in the same way. Hayem's fluid being used as a diluent. Smears of the suspension were prepared using the serum film technique of Harris (1956) and the smears were stained by the May-Grunwald-Giemsa method. Differential counts were made on 2000 cells from each suspension and the absolute number of cells per  $\text{mm}^3$  of bone marrow calculated for each main cell group.

The following are the mean values obtained for the total number of nucleated cells and for the number of cells in the main cell groups per  $\text{mm}^3$  of bone marrow in 10 normal rats (all figures including the standard deviation  $\times 10^3$ ).

The total number of nucleated cells amounted to  $2208.9 \pm 236$  cells. The absolute numbers of cells in the main cell groups were: Lymphocytes  $410.0 \pm 81.2$ . The majority of these cells were small lymphocytes but lymphocytes of all sizes are included in this group. Erythroid cells i.e. all nucleated precursors of the erythrocytes  $509.2 \pm 101.0$ . Myeloid cells including mature granulocytes and their pre-

unclassifiable within the main cell groups owing to severe damage.

The data here presented form the basis of experiments the purpose of which is to study the origin of the large population of lymphocytes found in the rat bone marrow. A report on these experiments will appear later. In the present study lymphocytes accounted for  $18.8 \pm 3.7$  per cent of the total number of nucleated cells. As a result of the previous exsanguination the blood content of the bone marrow was slight. The content of erythrocytes in the bone marrow averaged  $0.9 \times 10^6$  cells per  $\text{mm}^3$ . The number of erythrocytes per  $\text{mm}^3$  of blood in the animals used was  $7.7 \times 10^6$ . Consequently the blood contamination was about 0.117  $\text{mm}^3$  of blood per  $\text{mm}^3$  of bone marrow. The lymphocyte content of the blood in these rats averaged  $18.6 \times 10^3$  per  $\text{mm}^3$ . Thus only an insignificant portion of the bone marrow lymphocytes owe their presence to the blood content.

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Received 13 v 63 from Medicinsk Anatomisk Institut University of Copenhagen

(Comparative Endocrinology Wiley New York 1959) who stated that total pancreatectomy produces a mild type of diabetes in rabbits

The pancreatic rests in the subtotaly depancrealized diabetic rabbits have been studied. Pictures suggestive but not convincing of so called acino insular transformation have been obtained. Glycogen infiltration in hydropic  $\beta$ -cells has been shown

8 S Falkmer and P Anutson IS COBALT CONCENTRATED IN PANCREATIC ISLET TISSUE?

9 I Zamboni ELECTRON MICROSCOPICAL INVESTIGATION ON THE EARLY STAGES OF POLYOMA VIRUS INFECTION IN MICE

A pellet obtained by ultracentrifugation of medium from polyoma infected tissue culture and very rich in virus particles mostly attached to fragments of tissue culture cells was resuspended in a small quantity of fluid and subcutaneously injected in adult mice

10 minutes 40 minutes and 24 hours later subcutaneous tissue was excised from the injection area and studied with the Electron Microscope 15 minutes after the injection virus particles were observed in the cytoplasm of the tissue cells. They had been transported through the cell membrane as single units by pinocytotic activity or in clusters attached to the injected cell fragments by phagocytosis

After 45 minutes few particles were detectable intranuclearly whereas 24 hours later the nuclei of many cells were literally loaded with virus. The degeneration of the infected cells leads to a fragmentation of the elements their debris rich in virus particles are phagocytised by healthy cells with consequential spreading of the infection

10 A Bergstrand C G Bergstrand A Engstrom and A M Herrlin RENAL CHANGES DURING TRIDIONE TREATMENT

A nephrotic syndrome is a rare complication to treatment of epilepsy in children with Tridione. Experimental investigations also indicate that Tridione in large doses may have a nephrotoxic effect. In order to find out if slight and clinically non observed damage to the kidneys is a frequent complication we have investigated seven cases aged 15-17 years which have been treated with Tridione for periods between three months and eight years. Continuous renal examination showed only slight and transient haematuria or proteinuria in three of the cases

Light microscopy of renal needle biopsy material showed no glomerular or tubular lesions

Electron microscopy of the glomeruli showed wide variations in the appearance of the capillary epithelial cells. These are regarded as physiological variations and not as pathological phenomena

The investigation has not shown any signs of renal damage which could be assigned to the treatment

11 H Thorell LOCALIZATION AND DETERMINATION OF RESPIRATORY ENZYMES IN THE SINGLE LIVING CELL

With regard to the fundamental energy linked cell reactions e.g. the cell respiration our knowledge about the functions of the various cell structures has



## 5 B Lagerlof TRANSPLANTATION OF CHROMOSOMELY LABELLED LEUKAEMIA CELLS

A transplantation form of virus induced erythroleukaemia in the fowl can be obtained by inoculating the chick with intact leukaemia cells. The host's own bone marrow cells, which are the target for the virus, may, however, be infected by the virus released from the leukaemia cells and thereby undergo leukaemic transformation. In its final stage the disease thus developed would then be a combination of transplantation- and virus induced leukaemia.

The occurrence of transplanted and autochthonous leukaemia cells in the body has been studied by chromosome analysis. Hens the cells of which have 11 macrochromosomes and an unpaired fifth chromosome, were inoculated with leukaemia cells of cocks which have 12 macrochromosomes and paired fifth chromosomes. Three and four days after such inoculation the animals showed varying degrees of transplanted leukaemic proliferation in the bone marrow and in the spleens. The infiltration was in most cases due entirely to the proliferation of the transplanted cells. On the fifth day a few of the host's own cells were also recognized in the spleen and the bone marrow contained a mixture of autochthonous and transplanted leukaemic proliferation. It was concluded that during the first days the animals can develop a fairly pure transplantation leukaemia on which a virus induced component is subsequently superimposed.

## 6 G. Hultquist and J. Thorell TRIALS WITH PANCREAS TRANSPLANTATION IN RATS AND GUINEA-PIGS

Pancreas tissue from rats or guinea pigs with pancreatic duct ligation and containing islet tissue but no other remains of exocrine parenchyma except ducts was implanted in the anterior chamber of the eye. The transplantations were carried out both in the animals with duct ligation and in other members of the same litter. After one month islet tissue showing signs of growth was found in many of the rats - about 90 per cent of the case of autologous transplantation and about 30 per cent of the others. Much poorer results were shown by the guinea pigs in which the survival time was 9-10 days at the maximum.

The grafts always contained  $\beta$  cells showing a pseudo isocyanine reaction but  $\alpha$  cells were present only in some cases. In a few  $\alpha$  cells a positive reaction was obtained with Voigt's sulfide silver method.

Karyometry showed nuclear enlargement in both  $\beta$  and  $\alpha$  cells with maximum enlargement in 3-4 day grafts. The change gradually decreased and after 3-4 weeks the values corresponded to those of the normal pancreas.

## 7 G. Nathorst Windahl DIABETES IN PANCREATICTOMIZED RABBITS

Subtotal pancreatectomy in rabbits produces a relatively mild type of diabetes with glycosuria, polyuria, hyperglycaemia and an initial loss of weight. There is often an overproduction of ketone bodies but severe acidosis or coma does not develop. The animals are able to live for long periods with out insulin and yet often maintain an almost constant rather low body weight in spite of pronounced glycosuria.

Total or approximately total pancreatectomy however produces a severe diabetes with massive ketonaemia and ketonuria. Metabolic acidosis and coma develop in a few days unless insulin is given.

These results are at variance with the opinion of Greeley as cited by Houssay

acid fast Pollen was suspended in physiological saline and injected subcutaneously and intraperitoneally into rabbits (33 animals) The animals were killed 1-8 months later Foreign body granulomas developed around the injections but no epithelioid cell granulomatosis was observed The experiments provided no support to the hypothesis of a causal relationship between pollen and sarcoidosis

18 *L E Larsson* ENDOCARDIAL MYXOMA WITH MALIGNANT DEGENERATION

A woman without known rheumatic fever had for 3 years had progressive cardiac failure clinically interpreted as combined mitral and aortic disease During the last few months widespread skeletal lesions of malignant character were observed Necropsy revealed a myxomatous tumour at the fossa ovalis in the left atrium The endocardium in the atrium was thickened the mitral orifice was stenosed the mitral valves and chordae tendinae were rigid and thick and the aortic valves showed diffuse fibrous thickening The left lung showed a hilar tumorous nodule with peripheral extensions along the vessels Numerous metastases were seen in the heart wall in the skeleton and in the left ovary The atrial tumour exhibited the histological characteristics of an endocardial myxoma with sarcomatous degeneration The rest of the atrial wall the mitral and aortic valves chordae tendinae and the pulmonary veins showed marked fibrous thickening with a surface layer of anaplastic tumour cells The relations between the tumour and the valvular and pulmonary lesions are discussed

19 *L Olding* PERINATAL BACTERIAL INFECTIONS IN AN AUTOPSY MATERIAL

20 *C Lundmark* GRANULAR ATROPHY OF THE BRAIN—OBLITERATING ARTERIAL DISEASE

21 *C Lundmark* CARCINOID TUMOUR WITH METASTASES IN THE HEART AND AORTIC STENOSIS

22 *B Larsson* A CASE OF POSTANGIOGRAPHIC Th DEPOSITION AND PRIMARY SQUAMOUS-CELL CARCINOMA OF THE LIVER

A bilateral cerebral angiography had been performed with a total of 17 ml of Thorotrast Twenty seven years later the 53 year old male succumbed to a large necrotizing liver tumour squamous-cell in type Autops total body scintillography had shown no uptake in the region of the spleen before

and revealed a moderate and mainly post necrotic scarring Thorotrast deposition was demonstrated in liver and spleen with routine methods and autoradiography The bone marrow was in a reactive state A moderate anaemia was present There were no oesophageal varices

so far mainly been based on bulk analyses of isolated tissue fractions. The present paper describes some recent developments of the microspectrographic technique which permit quantitative *in vivo* assay of respiratory enzymes in localized areas of  $1.5 \mu$  in diameter in the single intact cell. The detectability is equivalent to 6000 molecules of cytochrome *b*. Soret spectra of both oxidised and reduced cytochromes can be demonstrated depending on whether the cell is in an aerobic or an anaerobic state. Measurements on the grasshopper spermatocyte and on isolated liver and kidney cells have given information about the metabolic state of the respiratory system even of the single structures within the living cell.

12 A. F. Åström EXPERIMENTS ON ALLERGIC ENCEPHALOMYELITIS

13 S. Szögi ASEPTIC MNINGOENCEPHALITIS AND MYOCARDITIS (PROBABLY COXSACKIE) IN A NEW BORN CHILD

14 S. Lundberg and O. Grøntoft A CASE OF BILATERAL SYMMETRICAL CALCIFICATIONS OF THE BRAIN

15 I. Bjersing and V. F. Borghin EOSINOPHILIA IN UTERUS

In recent years the authors have occasionally seen massive collections of eosinophilic leucocytes in the human uterine myometrium. Fifteen cases were studied in detail. Two groups were used for comparison. In one of the groups amputation of the uterus or cervix had been preceded by curettage or biopsy but not in the other. Eosinophilic granulocytes were observed—also in the controls—usually around vessels or between muscle bundles in the myometrium and showed no correlation with the amount or site of other inflammatory cells. No explanation can be offered for this abnormal eosinophilia which has as far as we know not been described before. The eosinophilia was slight and it was rare when the interval between the interventions was more than 3 weeks. Animal experiments in progress have shown absence of eosinophilic leucocytes in the uterus of spayed rats and eosinophilia in spayed animals treated with oestrogen, the degree of eosinophilia varying closely with the dose given.

16 G. Fichera and I. Hagerstrand LYMPHATIC CHANGES IN CHRONIC PULMONARY CONGESTION

A post mortem series of chronic pulmonary congestion (100 cases of mitral stenosis, 75 cases of pulmonary congestion because of hypertension or myocardial lesions) was studied. Compared with the controls (healthy lungs from 50 cases of different ages) fibrous mural thickening of the lymphatics was found at different levels (perivascular, peribronchial, interlobular and intrapleural) in 70 per cent of the cases of pulmonary congestion. This lymphatic fibrosis appeared to develop together with other pathological signs of chronic congestion and was probably not a primary lesion. The fibrosis was probably due to increased drainage of fluid.

17 I. Hagerstrand and F. Linell SARCOIDOSIS AND POLLEN

Experiments were performed to check the hypothesis put forward in recent years that sarcoidosis might be due to fir tree pollen. All of the pollen studied (different sorts of fir tree, pine, beech, oak, maple, alder, *Lycopodium*, timothy grass) proved

posterior diencephalic lesions. However these results are preliminary and must be confirmed by further studies.

The results support the view that there is no localized "sexual center" in the adult male rabbit. However posterior diencephalic lesions seem to induce alterations in spermatogenesis.

2 *P. Sourander* MORBID KRABBE

4 *I. Hagerstrand* THALIDOMID INDUCED MALFORMATIONS

5 *H. Sunzel and L. Zettergren* LESIONS IN THE LIVER AFTER SURGICAL OPERATIONS IN THE UPPER PART OF THE ABDOMEN

6 *L. Enerback* HISTOCHEMICAL REACTIONS IN CARCINOIDS

7 *F. Anulson* AUTORADIOGRAPHY OF PITUITARY CELLS IN ADRENAL-ECTOMIZED WHITE RATS AFTER THE INJECTION OF ADENINE-<sup>14</sup>C

Five male white rats weighing 150-200 grams were adrenalectomized. Observation times were 1, 2, 4, 8 and 12 days. Two hours before the end of each observation time the animals were injected with 0.05 mC adenine <sup>14</sup>C.

The pituitaries were fixed in neutral formalin, embedded in paraffin and sectioned serially. Autoradiography was performed with strip film technique and Kodak AR 10 emulsion. The unstained autoradiographic specimen was mounted in a glycerol medium and examined under a photo microscope with a micron graduated mechanical stage. Labelled cells were photographed in transmitted light and in phase contrast. The film was removed and the section stained with Tris PAS technique. With the aid of the graduated stage and the previously taken photographs the same labelled cells were localized in the stained section.

About 70 labelled cells within random fields in all pituitaries had been so localized. One day after adrenalectomy 70 per cent of labelled cells were small chromophores. With increasing time after adrenalectomy their percentage gradually diminished whereas the number of labelled large vacuolized chromophores and amphophiles increased, reaching almost 50 per cent 8 and 12 days after adrenal ectomy. Very few well granulated basophiles were labelled. The results point at the chromophobe cell as the site of new production of ATCH and it was suggested that these chromophores gradually turned into vacuolized forms and so called amphophiles.

8 *P. Lunin* THE CARCINOGENIC ACTION OF COMPLEX IRON PREPARATIONS

9 *H. Röckert and L. Zettergren* LESIONS CAUSED BY BARIUM CONTRAST MEDIA

10 *I. Angerall and S. F. Fagerberg* SKIN BIOPSY IN DIABETES

Since the beginning of 1961 punch biopsies from the skin on the dorsal part of the basal phalanx of the 4th toe of diabetics have been examined. All specimens have been fixed in 10 per cent neutral formalin solution, about half of them being

# TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

*Meeting April 7, 1962*

## 1 *I.-B. Schnürer and S. Stattin* THE VASCULAR SUPPLY OF TENTORIAL MENINGIOMAS

In some papers dealing with tentorial meningiomas vessels have been described seemingly arising from the carotid siphon. This prompted an investigation on the topography and distribution of these vessels performed on autopsy specimens with the aid of a dissection microscope. The vessels were successfully injected and dissected in about 15 of the specimens. The number and course of the major branches were found to be rather constant. Thus there are two main branches, one arising from the infero-lateral aspect of the so-called horizontal portion of the siphon, the other from the upper posterior aspect of the convexity of the proximal portion of the siphon. In most cases both main stems give off tentorial branches; in some only one of them does. The tentorial branch of the lateral main stem in most cases runs directly upward, enters the tentorium and continues backward near the free margin, sometimes up to the summit. The tentorial branch of the dorsal main stem is usually smaller and runs backward in the tentorium near its attachment to the petrous bone. These tentorial vessels are most often but not always too small to be visualized at angiography, but they may hypertrophy when contributing to the vascular supply of meningiomas and vascular malformations and thus be of angiographic and neurosurgical importance.

## 2 *C. Ahren* EFFECT OF DIENTEPHALIC LESIONS ON TESTES IN ADULT RABBITS

Diencephalic lesions were produced in 248 adult male rabbits with the aid of Rastrom's X-ray stereotaxic instrument in medial parts of the diencephalon from the anterior medial preoptic area to the rostral border of the mesencephalon. The animals were allowed to survive for 6 weeks; half of these were subjected to repeated subcutaneous injections of formalin during the final 3 weeks. On the basis of the size, localization and symmetry of the lesions determined histologically from serial sections of the diencephalon 140 animals were segregated into 10 groups (each about equally divided as to formalin treatment). The median eminence and pituitary gland were histologically intact in all animals.

*Results.* Some animals showed gross testicular atrophy not correlated to any particular lesion. Severe atrophy was only observed with formalin treatment, probably a non-specific stress reaction. There were no significant differences in the mean testicular weight in the 10 groups as compared with the control animals. Histological signs of altered spermatogenesis in some animals suggest disturbances in secretion of FSH. Such changes were most frequently observed in animals with

# TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

*Meeting December 1, 1962*

## 1 L. Santesson ON THE CLASSIFICATION OF OVARIAN TUMOURS

At an international conference in Stockholm the following proposal was made  
The Conference recommends that the common epithelial tumours of the ovary be classified in the following manner

### 1 Serous cystomas

- a) Serous papillary cystadenoma benign (Figs 69 and 71)
- b) Proliferating serous papillary cystadenoma without stromal invasion (possibly malignant) (Figs 72 and 75)
- c) Serous cystadenocarcinoma all grades (Figs 78 and 81)

### 2 Mucinous cystomas

- a) Mucinous cystadenoma benign (Figs 87 and 90)
- b) Proliferating mucinous cystadenoma without stromal invasion (possibly malignant) (Figs 91 and 92)
- c) Mucinous cystadenocarcinoma all grades (Figs 93 and 95)

### 3 Endometrioid tumours

- a) 1  
b) Proliferating endometrioid adenoma and cystadenoma (possibly malignant) (Fig 102)
- c) Endometrioid adenocarcinoma all grades (Figs 77 and 105)

### 4 Undifferentiated carcinoma (cell type unknown)

[The numbers in parenthesis refer to figures published in "Tumors of the Ovary and Fallopian Tube" by Hertz & Gore II (Atlas of Tumor Pathology Section IV Fascicle 33 Tumors of the Female Sex Organs Part 3 Armed Forces Institute of Pathology Washington 1961)]

Usually in any given tumour only one type of epithelial cell is dominating. Occasionally when there is a mixture of different cell types the predominant type should determine the diagnosis.

## 2 S. Falkmer, F. Knutson and G. E. Vargl FURTHER STUDIES ON THE COBALT CONCENTRATING ABILITY OF PANCREATIC ISLET TISSUE

The recent discovery by quantitative scintillation determinations using  $^{58}\text{Co}$  that isolated islet tissue has approximately the same high ability as the liver to con-

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<sup>1</sup> The existence of a blank space after 3 a) is due to the fact that there is as yet no agreement as to whether some cases of ovarian endometriosis may properly be classed as true tumours or all must be regarded as simply displaced or ectopic endometrium.

fixed also in 4 per cent basic lead acetate solution. The skin specimens were cut into 4  $\mu$  serial sections consecutive sections being stained with haematoxylin eosine van Gieson haematoxylin Hansen and MacManus' periodic acid Schiff. So far we have examined biopsy specimens from 50 male and female diabetics of varying ages and from 35 non diabetics.

All but one of the diabetics exhibited dermal microangiopathy in the form of wall thickening and reduction in the lumen due to deposition of a strongly PAS positive substance which in van Gieson stained sections was a homogeneous mildly picrinophilic material. Some vessels displayed hypertrophic hyperchromatic nuclei between PAS positive fibrils of the basement membrane. The diabetic microangiopathy is usually most pronounced in the capillaries surrounding the sweat glands and in the papillae. The larger the vessels the more difficult it is to find characteristic lesions.

All but one of the controls showed in general a suggested or slight PAS positive reaction.

The facts that diabetic microangiopathy has now been encountered in the skin constitutes additional evidence that it occurs throughout the organism.

chicks with a late onset of the disease dying at an age of more than one month had a significant increase of the gamma globulin fraction. This probably contains viral antibodies and may thus be responsible for the fact that the virus concentration of the plasma from leukaemic chicks decreases with increasing age of the birds. Young chicks with marked viraemia had normal amounts of gamma globulin. Chicks even young ones with the subleukaemic anaemia had high gamma globulin fractions which taken together with the lymphoid hyperplasia and the signs of haemolysis seems to indicate an immunologic cause of the disease.

#### 6 T Saldeen EXPERIMENTAL INVESTIGATION OF CANCER SPREAD TO THE LIVER IN RATS WITH LIVER CIRRHOSIS

Moderate liver cirrhosis induced by administration of carbon tetrachloride to rats increased the percentage of secondary intrahepatic growths following intraportal injection of tumour cells from Rous rat sarcoma. The number of tumour nodules in the liver was higher when the interval between the last injection of carbon tetrachloride and the injection of tumour cells was 4 or 6 weeks than when it was 2 weeks. A positive correlation was found between the extent of fibrosis in the liver and the number of tumour nodules.

After injection of tumour cells into a tail vein tumour nodules were seen in most cirrhotic but in no normal livers.

Liver cirrhosis had no effect on the growth of the tumour after direct intrahepatic injection of the tumour cells suggesting that the vascular changes in the liver are responsible for the increased number of intrahepatic secondary growths.

A possible increase in the number of tumour cells trapped in the cirrhotic livers can however hardly explain the higher percentage of takes in the liver. Another possibility is that the narrowing and obliteration of the smaller vessels prevented the tumour cells from coming into contact with Kupfer's cells.

#### 7 J Zajicek NEEDLE BIOPSIES AS A METHOD TO OBTAIN HUMAN TUMOUR MATERIAL FOR BIOCHEMICAL AND BIOLOGICAL INVESTIGATIONS

#### 8 P S Persson CYTOLOGIC DIAGNOSIS OF SUBACUTE AND CHRONIC THYROIDITIS

Thin needle punctures were carried out in 175 patients with goitres. The smears were stained according to the May Grunewald Giemsa method. Of 9 patients with the clinical picture of subacute thyroiditis 6 had the cytologic findings of subacute thyroiditis and 2 a suspected one. No definite diagnosis could be made in 1 patient. In 42 patients the cytologic diagnosis was chronic thyroiditis. In 24 patients the diagnosis was verified by histopathological examination, the rest had a typical clinical picture. There were no cytologic differences between various kinds of chronic thyroiditis.

The cytologic findings of subacute thyroiditis vary with the phase of the disease and present the following features: (1) marked degeneration of the follicular epithelial cells; (2) proliferation of the follicular epithelial cells in the late phase of the disease; (3) foreign body giant cells; (4) collections of polymorphonuclear leucocytes, histiocytes and mononuclear inflammatory cells.

The cytology of chronic thyroiditis is characterized by (1) proliferation and to a lower degree (2) degeneration of the follicular epithelial cells; (3) collections



centrate cobalt (Acta Endocr 42 1963) prompted us to try further localization of the cobalt in the pancreatic islets. As conventional autoradiography had shown that the accumulated cobalt was easily dissolved from the tissue during fixation and embedding procedures we had to modify the histochemical and autoradiographical methods.

The isolated islet tissue of the marine teleost *Cottus scorpius* was cut in a microtome cryostat. Autoradiography was performed without the sections coming in contact with water. Other islets were gassed with hydrogen sulphide, sectioned in the microtome cryostat and subjected to a modified sulphide silver procedure to detect precipitated cobaltous sulphide after extracting other heavy metal sulphides. The injected cobalt was localized to the islet parenchyma and not to the stroma. By comparing the autoradiograms and the sulphide silver sections with adjacent sections stained with aldehyde fuchsin (for the visualisation of the B and A cells) it was possible to show that the injected cobalt was confined only to the cells in the dark central region of the sculpin islets containing the B cells and the argyrophil A cells.

#### ■ B Stenqvist GROWTH CURVES, HISTOLOGICAL APPEARANCE AND VIRAL TITRES OF ROUS SARCOMAS. A COMPARISON BETWEEN PROGRESSIVE AND REGRESSIVE GROWING TUMOURS

#### 4 J Thorell THE PLACENTAL TRANSFER OF INSULIN ANTIBODIES

The insulin antibody titre was assayed by measuring the capacity of serum to bind insulin- $^{125}$ I. After incubating the sera with insulin- $^{125}$ I for 5 days at 4°C the antibody-bound insulin was separated from the unbound insulin by paper electrophoresis according to Berson & Yalow. The antibody titres were assayed in blood obtained from the umbilical cord and from the mother on the same occasion.

In pregnant guinea pigs with relatively low insulin antibody titres the insulin binding capacity of the foetal blood was usually double that of the maternal blood at term.

The titres of three newborn children of insulin treated diabetic mothers were about equal to their mothers titres.

This placental transfer of antibodies may be of some importance for the foetal insulin metabolism. Further studies on this subject are in progress.

#### 5 P Sundelin and B Lagerlöf DISORDERS INDUCED BY MYELOID LEUKAEMIA VIRUS

Myeloid leukaemia virus can induce several different diseases in the fowl. The type of disease induced has been shown earlier to depend on the age of the bird and the amount of virus inoculated. An investigation into the pathogenic effect of the myeloid leukaemia virus revealed that our virus strain has a pathogenic spectrum similar to that described earlier and induces myeloid leukaemia, visceral lymphomatosis and kidney tumours. However, in our material an additional form of leukaemia, a subleukaemic anaemia, was observed. This form of the disease is characterized by lymphoid hyperplasia and occasional areas of necrosis in the bone marrow, a severe anaemia, a comparatively low number of leukaemic cells in the peripheral blood and siderosis of the spleen indicating haemolysis.

An electrophoretic investigation of the plasma from leukaemic birds revealed that

11 *A Cederberg and G Ostberg* OXYGEN INTOXICATION

A girl of eighteen after some days of sore throat suddenly developed pain in the right side of the chest and heavy respiratory distress. On the third day she was taken to hospital where blood was found in the right pleura. She was given transfusions and treated with drainage of pleura later on tracheotomy and artificial respiration with increasing partial pressure of oxygen up to 52 per cent in the last hours. She died on the tenth day. Clinical diagnosis: Spontaneous haemo pneumo thorax.

At autopsy the lungs which weighed 830 and 870 grams were strikingly heavy and firm. No rupture or bleeding was found on the lung surface. Microscopically a dense fibrinous exudate filling the alveoli of right lower lobe was seen. Other parts showed widespread formation of hyaline membranes on alveolar septa.

Pulmonary hyaline membranes of adults are rare. They seem to occur without any certain relation to other diseases. The rôle of oxygen treatment has been discussed. Possibly hyaline membrane formation can be induced by thoracic surgery. Our case might have a similar mechanism with collapse of the right lung and compression of the left at spontaneous haemo pneumothorax. Delay of treatment may have played a rôle.

12 *J Söderström* SPLENIC RUPTURE IN INFECTIOUS MONONUCLEOSIS

About 50 cases of splenic rupture in infectious mononucleosis are reported. The histological picture is described on the basis of a personal collection of 6 cases. Three of the 6 patients had died and 3 had been operated upon with success. The spleen showed intense proliferation of large mononuclear cells above all in the red marrow but also in the capsule and the trabecular network that had thereby become loose which probably facilitated the rupture. The cellular proliferation in the vessels was noteworthy. Large round cells were seen in the adventitia of the arteries and subintimally in the veins. Other organs showed similar changes. The picture was the same in all the cases studied and coincided with descriptions of earlier reported cases.

The most important disease to be considered in the differential diagnosis is leucosis. Careful pathological examination of the characteristic—though not pathognomonic—histological picture together with clinical and laboratory data enable a firm diagnosis of mononucleosis infectiosa.

13 *A Rausing* GLIOMA INDUCED BY FOREIGN BODY

A 55 year old woman with bilateral symptoms of parkinsonism since 1951. 1956 pallidotomy right side leaving two spirals of tantal in the brain. 1959 left sided thalamotomy no foreign bodies left behind. No relapse on left side, speedy recurrence on right side and progressing symptoms of increased intracranial pressure until death in 1962.

*Autopsy* small-orange sized cyst in right sided field of operation. Two metal spirals were found in its wall. Walnut sized tumour in the right occipital lobe. Brownish discoloration in the left sided field of operation. *Histology* tissue from right sided cyst wall and tumour and left sided lesion showing malignant glioma. No continuity proved between the different sites but made probable by strongly reactive picture in the tissues in between.

A growth with this histological picture must have arisen after the first operation. *Oppenheimer et al* have succeeded in creating spindle-cell sarcomas by subcutaneous

of lymphocytes lymphoblasts plasma cells reticulo endothelial stem cells and phagocytes Epitheloid cells may sometimes be seen

Difficulties in the differential diagnosis exist between thyroiditis and special types of thyrotoxicosis and malignant thyroid diseases

### 9 L G Wiman SULPHYDRYL CHEMISTRY AND FLUORESCENCE MICROSCOPY IN EXFOLIATIVE PULMONARY CYTOLOGY

Two new cytochemical staining procedures for exfoliative cytology are introduced With one method first published in 1959 by the author a high concentration of proteinbound sulphhydryl groups is demonstrated in exfoliated tumour cells In ordinary light microscopy these cells stain intensive blue and can be easily distinguished from surrounding normal cells which appear as pale shadows With a second method (1962) the ability of tetracycline to fluoresce in ultraviolet light is used to display tumour cells In a patient treated with tetracycline the substance is concentrated in tumours especially According to their conspicuous yellow green fluorescence exfoliated malignant cells can be easily identified in smears of sputum bronchial secretions aspirates from pulmonary tumours and pleural fluids If unfixed smears from patients not treated with tetracycline are immersed in a tetracycline solution a strong yellow fluorescence is induced in the tumour cells Upon the same material Bertalanffy's method was tried using fluorescence microscopy after staining with acridine orange Hereby certain malignant cells got a bright red fluorescence on account of a high content of RNA in cytoplasm and nucleolus which makes them easy to find during screening work However malignant cells exfoliated from a highly differentiated squamous cell carcinoma often have a low content of RNA For this reason they get a faint olive green or orange red fluorescence and may therefore be overlooked in the microscope This and some practical disadvantages gives the method a limited value in diagnostic cytology The results of all methods above has been correlated to and compared with the Papanicolaou staining procedure

### 10 A Gydell I Juhlin and J G Norden NOCARDIOSIS

A 41 year old female acquired an auto immunologic haemolytic anaemia and was treated with increasing doses of steroids (average dose during 1 year 29 mg of Deltacortril® per day) After 8 months she got fever and chest roentgen showed infiltrations which later cavitated No acid fast bacilli were found guinea pig inoculation was negative but nocardia was cultivated from bronchial secretion After heavy medication with penicillin the patient improved transiently but then she became worse with deterioration and epileptiform seizures Increasing sputum blocked the air passage On treatment with Elkosin® (sulphaisodimidin) the chest roentgen picture became almost normal and nocardia disappeared from the trachea But the blood values decreased and finally she became unconscious and died

*Autopsy* In the lungs solid yellow foci and a system of cavities were noted The kidneys showed several yellow streaks and a larger necrotic focus which continued into a perirenal abscess with greenish thick necrotic material Such abscesses were also found in all parts of the brain and in musculature (neck and leg) Nocardia could be demonstrated in cultures with material from brain and kidney but in no other organ

The changes of the oral mucosa were the same as in earlier reported cases. The polydysplastic case showed a peculiar discharge of well defined parts of connective tissue demarcated by ingrowing epithelial strands.

In the tooth germs heavy disturbances in the enamel formation were found such as metaplasia of the enamel epithelium with many epithelial whorls. In micro radiograms these appeared as globular irregularly mineralized structures. The enamel was considerably thinner than normal. In the case where the teeth had erupted they showed severe hypoplasias similar to those clinically seen in amelogenesis imperfecta. In all the cases the tooth germs showed the same disturbances. It is concluded that amelogenesis imperfecta may form a stigma in the reported forms of epidermolysis bullosa hereditaria.

#### 18 H Larsson ON MALIGNANT LIVER AND BILE DUCT TUMOURS IN CASES EXPOSED TO THOROTRAST AND AMONG WINGGROWERS WITH LONG-STANDING EXPOSITION TO ARSENIC

The two groups were briefly presented as available in the literature. The non epithelial tumours were mainly discussed carcinomas being further referred to elsewhere. Some differences between the radioactive  $\text{ThO}$  and the chemically toxic  $\text{As O}_3$  were mentioned. The Th sarcomas appeared after an average time of 16 years and the As tumours after 20 years. The Tumour Development Time (TDT) in these human series represents Tumour Draht Time, in experimental series a Tumour Detectable Time might be registered.

It is obvious that different substances can induce similar tissue responses. In the present two sarcoma groups there is one cellular main response and one terminal lesion.

Human tumours possibly exogenously induced and certain characteristics in the response of some animal species to carcinogenic influence were considered to present a tentative basis for a further study of common denominators in the process of malignant development.

#### 19 S Håksten RENAL CELL CHANGES AFTER INFUSION OF LOW VISCOSITY DEXTRAN

During the last year a great number of cases with changes in the renal tubules of the type known as osmotic nephrosis have been found at post mortems at the Karolinska sjukhuset. 31 cases featuring such changes have been diagnosed and out of these 27 had been given so called low viscosity dextran (average molecular weight 40000) in amounts ranging from 100 to 4400 cc. The other four cases had been given rather large doses of various hexoxis. It has not been possible to determine whether the tubular changes are accompanied by disturbances of function. In spite of the fact that the commonly used type of dextran is not osmotic —

— a low viscosity dextran seems striking

#### 20 S Sjögt ISOLATED TOXOPLASMA MYOCARDITIS IN A CHILD

After  
the  
beer

implantation of tantal plates. It is suggested that neuroglia may give a malignant response to a similar stimulus.

#### 14 C Lundmark A FATAL CASE OF VARICELLA

#### 15 I Olding EPIDERMOLYSIS BULLOSA LETHALIS SOME MORPHOLOGICAL OBSERVATIONS

Sections of the skin from four infants who all died within nine weeks after birth and who had all been affected with epidermolysis bullosa lethalis were examined by conventional histological methods. Sections were stained with hematoxylin and eosin and Weigert's elastin stain. The argyrophile reticulum fibers were visualized by Landau's method. Sections of skin from dead infants at different ages and without skin diseases were used as controls.

The early changes of epidermolysis bullosa lethalis were multiple intracellular vesicles in the basal layer of the epidermis. The subepidermal elastin plexus was practically absent but this was also the case in the controls. This coincides with the observations of Dick (J. Anat. 81: 201, 1947) who has shown that the subepidermal elastin plexus is practically absent in the normal skin in infancy. On the other hand the reticulum fibers in the uppermost dermis were well developed both in the cases of epidermolysis bullosa lethalis and in the controls. These reticulum fibers were prominent also at the margins of recent bullae where the basal layer showed a marked vesicular degeneration.

#### 16 B Robertson REPARATIVE PHENOMENA IN 'HYALINE' MEMBRANE DISEASE

In an autopsy material of 117 neonatal deaths varying in age between 6 hours and 6 days all displaying pulmonary hyaline membranes proliferative and reparative phenomena in the alveolar epithelium were observed in cases surviving more than two days. This agrees with the observations recently reported by Boss & Craig (Pediatrics 29: 890, 1962). Similar changes in the alveolar epithelium were also observed in four other autopsy cases, premature infants varying in age between 10 and 24 days. The clinical picture in these four cases had been that of idiopathic respiratory distress since shortly after birth. Histological sections from the lungs displayed thickened alveolar walls containing fibroblast like cells and excess of collagen and reticulin. No hyaline membranes of the usual type were observed in these sections although small amounts of hyaline material were present in some alveoli.

These findings reflect a process of repair which indicates previous damage to the alveolar walls possibly connected with the development of hyaline membranes. But since the author has also observed similar changes in the alveolar walls of 2-3 week old neonates without typical clinical history of idiopathic respiratory distress it is too early to draw any definite conclusions. A clinical analysis of these cases is in progress.

#### 17 T Arnell & Bergenhoff and O Olsson EPIDERMOLYSIS BULLOSA HEREDITARIA A HISTOPATHOLOGICAL STUDY OF TIBIAL AND ORAL MUCOSA IN THREE CASES

Demineralized sections and microradiography in ground sections from the jaws of patients with a dysplastic and lethal form of ebh were studied.

The changes of the oral mucosa were the same as in earlier reported cases. The polydysplastic case showed a peculiar discharge of well defined parts of connective tissue demarcated by ingrowing epithelial strands.

In the tooth germs heavy disturbances in the enamel formation were found such as metaplasia of the enamel epithelium with many epithelial whorls. In micro-radiograms these appeared as globular irregularly mineralized structures. The enamel was considerably thinner than normal. In the case where the teeth had erupted they showed severe hypoplasias similar to those clinically seen in *amelogenesis imperfecta*. In all the cases the tooth germs showed the same disturbances. It is concluded that *amelogenesis imperfecta* may form a stigma in the reported forms of *epidermolysis bullosa hereditaria*.

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#### 20 S. Szögi ISOLATED TOXOPLASMA MYOCARDITIS IN A CHILD

Necropsy report of a 5 year-old boy, who was found dead in his bed. The boy had often had infections of the respiratory tract. During the previous 6 months he had been fairly depressed.

The only gross finding of interest at autopsy was enlargement of the heart (wt 120 gm). Microscopic examination however revealed focal myocarditis with accumulations of lymphocytes around necrotic muscle fibres. An intracellular thin walled pseudocyst packed with crescent shaped or round parasites about 3-4 microns long and with distinct nuclei rich in chromatin was also seen. Its appearance was that of a *Toxoplasma* pseudocyst. No inflammatory reaction was seen around the pseudocyst. The groups of lymphocytes in the myocardium probably marked the sites of ruptured pseudocysts.

*Toxoplasma* myocarditis occurs in both an acute and a chronic form. The prognosis of the latter is much better than that of the former. Cases of myocarditis of unknown origin may be due to *Toxoplasma*. It is however difficult to distinguish between *Encephalitozoon*, *Sarcocystis* and *Toxoplasma*.

## GROWTH CURVES, HISTOLOGY AND VIRUS TITRES IN ROUS SARCOMAS

*A Comparison Between Progressively Growing and Regressing Tumours*

By

BJÖRN STENKVIST<sup>1</sup> and JAN PONTÉN<sup>2</sup>

Received 13 VII 62

Unlike many other tumours Rous sarcomas have a definite tendency to regress spontaneously. The percentage of regressing tumours increases with lowering of the virus dose. This interesting feature has never been the subject of more extensive studies and the underlying mechanisms are far from understood. The aim of this study is to obtain some basic data such as the shape of the growth curve of the tumours, the virus content, and the histology. The experiment is in essence a comparison between lethal progressively growing tumours and regressing tumours induced by the same virus dose.

### MATERIALS AND METHODS

**Animals** 36 non inbred White Leghorn cockerels 24 days old at the time of RSV inoculation.

**Virus** A 10 per cent saline extract of solid Rous tumours diluted  $10^{-1}$ . The RSV strain was originally obtained from the Rous Institute.

**Inoculation** Both wings were inoculated subcutaneously in the upper part of the wing.

**Growth curve** The tumours were palpated according to a scheme devised by Pontén (1957) starting on day 6. Three different diameters of the tumour (length, breadth and thickness) were measured with a caliper. The tumour volumes were calculated as spheres with the mean of the above three measurements in millimeters taken as the diameter.

For Nembutal anaesthesia a piece was used for

After the experiment the animals whose tumours had regressed and a selection of specimens from the animals which died from progressive tumour growth were thawed and homogenized with 3 ml of phosphate buffered saline (PBS) in a cooled VirTis microcup for 60 seconds. After centrifugation at 5000 rpm the supernatant was pipetted off and part of it diluted to  $10^{-2}$ . 0.2 ml of



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**Virus** A 10 per cent saline extract of RSV strain was origin 103 The RSV

**Inoculation** 0 cutaneously in both wings i.e.

**Growth curves** The inoculation sites were regularly palpated according to a scheme devised by Bryan (1956) starting on day 6. Three different diameters (of the tumour (length breadth and thickness) were measured with a caliper. The tumour volumes were calculated as spheres with the mean of the above three measurements in millimeters taken as the diameter.

**Biopsy** Small pieces of tumours were surgically removed under Nembutal anaesthesia. Care was taken to avoid necrotic areas. One half of the piece was used for histology the other was kept frozen and used for virus titration.

**Virus titration** The tissue specimens were kept frozen at  $-65^{\circ}\text{C}$ . After the experiment was concluded the specimens for the

#  
t  
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1. A supernatant was pipetted off and part of it diluted to  $10^{-7}$  0.2 ml of

<sup>1</sup> This work was supported by the Swedish Cancer Research Council and by USHS research.

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Pa. (USA)

the undiluted extract and the extract diluted to 10<sup>-8</sup> was injected into the wing webs of 2 day old test chickens. Each chicken receive one inoculation. Ten chickens were used for each dilution. The chickens used for bioassay were palpated according to Bryan (1956), and the latent time until a palpable tumour developed was noted.

The individual "titres" were then calculated according to a method described previously (Ponten 1956). The present titration differed from the former in that only one inoculation site per chicken was used and that only two different dilutions were injected. A correction was made for the weight of the homogenized sample. The intersection between the regression line and the dose axis corresponds to the titre value.

## RESULTS

**Growth curves.** The response of the 36 experimental chickens with respect to the length of the induction time was quite uniform. The mean induction time was 10.5 days. At 66/72 inoculation sites a tumour developed. 24/36 animals with 45 wing tumours died within 32 days due to rapidly growing tumours in one or both wings. 7/36 animals died between days 32 and 84. The tumours in these animals varied in size considerably, but they all eventually regressed.

4/36 animals had tumours that regressed and were still alive 327 days after inoculation with no other visible remnants of their tumours than small scars. 1/36 animals was consistently negative in both wings.

Forty-five progressively growing and lethal tumours from the group that died within 32 days were compared to 7 regressing tumours from four surviving chickens.

Already a superficial analysis made two facts clear. The host had a decisive influence on the growth behaviour of the tumours. This was attested by the fact that no animal carried one progressing and one regressing tumour at the same time. The seven regressing tumours were not distributed at random but were all found in four animals. The other fact was that in each animal one tumour was always "leading". The tumour that started earliest was at all times larger than the tumour in the other wing that started later.

The material has been divided into the following three categories:

1) *Progressive tumours*. These tumours are defined as growths which never showed a decreasing size and which led to the death of the host within 32 days.

The group was divided into two subgroups, the "largest progressive tumours" and the "smallest progressive tumours". In each animal the largest tumour, which always made its appearance earliest, was assigned to the former group, whereas the tumour of the other wing was assigned to the latter group.

2) *Regressive tumours*. These tumours are defined as growths which after having reached a certain volume decreased until no remnants (except a small scar) remained. These tumours went through a stationary phase between days 23 and 27. The animals were still alive 327 days after inoculation.

The group was divided into subgroups, "largest regressive tumours" and "smallest regressive tumours" analogously to the progressing tumours

3) *Irregular tumours* These tumours occurred in animals dying between days 32 and 85. The tumours all eventually regressed after having shown fluctuations in size. The cause of death of the animals remains unknown since no autopsies were performed.

The tumours of this group were subdivided into "largest irregular tumours" and "smallest irregular tumours" according to the same principle as the two other groups of tumours.

The crucial period during which it seemed to be determined whether a tumour would regress or not, was around 23-27 days after virus inoculation. Before this period all tumours increased rapidly in size. Within and after this period only the progressively growing tumours continued to increase in volume. The increase seemed, however, to be slowed down around day 23-27. The regressing tumours went through a stationary phase during the critical period around day 23-27 after which they regressed rapidly.

Fig. 1 is a comparison between "largest progressive tumours" and "largest regressive tumours". It is seen that during the progressive phase the regressive tumours were on the average somewhat smaller than the progressive tumours. The growth rate as expressed by the progression coefficient was essentially similar (0.15 and 0.13). Of interest is the rapidity by which the regression took place. The rate of regression (coefficient  $-0.15$ ) was comparable to the rate of progression in the same group (coefficient  $0.13$ ).

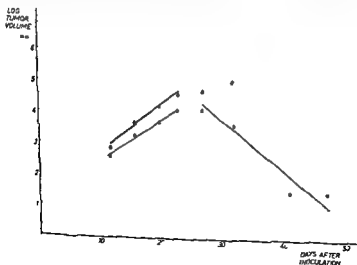


Fig. 1

Graph with curves of "largest progressive tumours" and "largest regressive tumours". See Text.

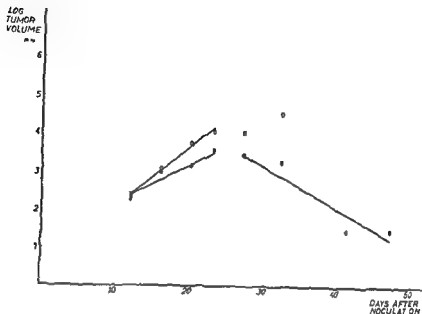


Fig 2

Growth curves of smallest progressive tumours and smallest regressive tumours  
See Text

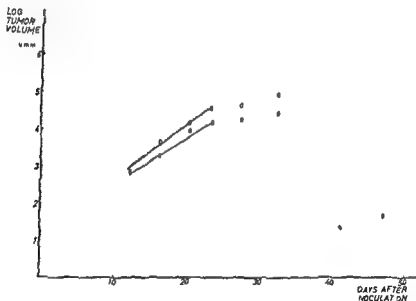


Fig 3

Growth curves of largest progressive tumours and largest irregular tumours  
See Text

Fig 2 is a comparison between smallest progressive tumours and smallest regressive tumours. Again the regressive tumours were somewhat smaller on the average than the progressive tumours at least after day 19. The growth rate was approximately the same (coefficient 0.16 and 0.11) and the rate of regression numerically equal ( $-0.11$ ) to the rate of progression in the same group.

Figs 3 and 4 are comparisons between the progressive tumours and

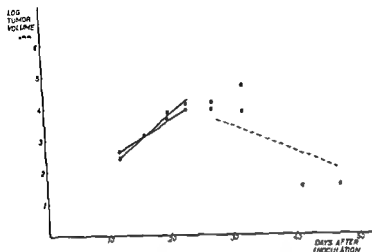


Fig 4

Growth curves of "smallest progressive tumours" and "smallest irregular tumours"  
See Text

the 'irregular tumours of group 3. During the progressive phase no significant difference was found between the two groups. Regression was seen from the wide scatter of the means was more irregular than in the 'regressive' group.

**Bioassay of virus.** See Table 1. Eight samples from 6 different progressive tumours were removed between days 13-27. The "titres" varied from 4.1 to 9.3 (mean 7.2).

Seven samples were removed from regressive tumours during their progressive growth phase. The biopsies were made on days 13 or 16. The "titres" varied from 2.4 to 9.0 with a mean of 5.6.

Nine samples were analyzed from 7 regressive tumours after their progressive growth terminated, *i.e.*, after day 23. The biopsies were taken on day 24, 27, 34. The "titres" varied from 0-4.5.

Six samples were taken from one of the animals which carried "irregular tumours." all showed high "titres" irrespective of the growth phase of the tumour. The values varied from 4.5 and 8.2 with a mean of 6.3.

**Histology.** During progressive growth the tumours displayed the well known features of Rous sarcomas (Fig 5). The tumours varied somewhat: most tumours were mainly of the spindle-cell type. A few, however, were dominated by large macrophage like cells rich in cytoplasm. There were always areas with necrosis, haemorrhage, and lymphocyte infiltration. No difference was noted between tumours that were to regress and those that would continue to grow progressively.

TABLE 1  
Virus Titres (see text) in Biopsies of Rous Sarcomas in Different Stages of Growth

	Chick number	Titre value on day after inoculation															
		13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Progressive	R* 9505 L			93												87	
Tumours	R 64																
	I 91															72	
	R 41																
	I															77 86	
Regressive	R 53															45	
	I 40																
Tumours	R 90																
	L 66																
	R 81																
	I 24																
	R 35																
	I																
Irregular	R 68																
	I 68																
	R 55																
	I 59																

\* R = right wing web, I = left wing web

24  
17

Regression

0  
26  
0

45  
82

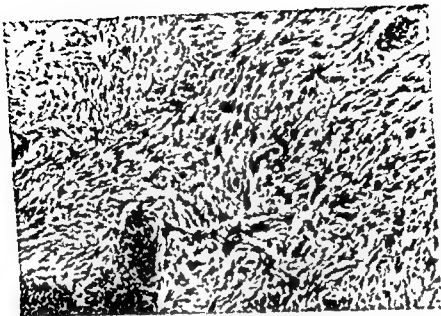


Fig 5

Progressively growing Rous sarcoma of typical fibrosarcomatous appearance H & E.  $\times 120$



Fig 6

Intermediate stage of regression. To the left partly necrotic Rous cells with pyknotic nuclei. The middle part of the figure is occupied by a band of lymphocytes to the right fibrous tissue H & E.  $\times 120$



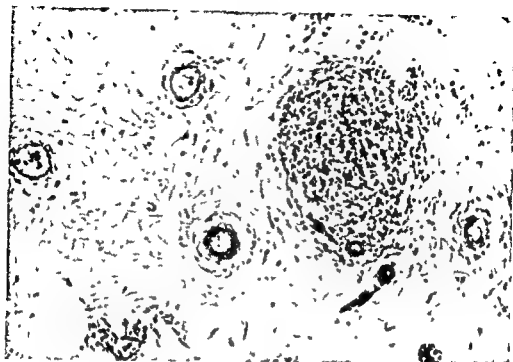


Fig 7

Advanced stage of regression. Fibrous tissue with some vessels of fine calibre and a lymphocytic focus. H & E  $\times 120$

The histologic examination of the regressing tumours failed to show a specific pattern. The advanced stage was characterized by fibrous tissue with scattered foci of lymphocytes (Fig 7). In an intermediate stage (Fig 6), the tumours were surrounded by a fairly dense band of inflammatory cells, mainly lymphocytes and macrophages. Outside this zone, connective tissue was found. Even after the tumour was grossly regressing, areas with viable sarcoma cells, some of which were in mitosis, were still seen.

#### DISCUSSION

The objective of the present study was to obtain some basic data about regressing Rous tumours.

The most important fact established was the correlation between the state of growth and the content of virus. The variation between the individual biopsies was considerable. It could, however, be established that a progressively growing tumour, in general, contained far more infective virus than a regressing one. In fact, in 4 samples from regressing tumours no infective virus at all could be recovered. This finding would seem to parallel findings in an ascitic Rous sarcoma where a similar correlation between the state of growth and the titre of cell-associated virus was found (Ponten 1956).

The significance of the correlation is questionable. It may represent a purely secondary phenomenon. The slowly growing or degenerating

cells in a regressing tumour may not be able to produce virus efficiently. On the other hand, the drop in infective virus titre may be the cause of the regression. Recent experiments with sex-marked cells (Ponten 1962) have indicated that virus spread and new infection of normal cells may be an important factor for the maintenance of progressive growth in a Rous tumour. If the cells stop producing virus, or if a substantial proportion of the infective particles are neutralized by antibody, regression will eventually result. The finding of little or no virus in the regressing tumours is thus consistent with the hypothesis (Ponten in preparation) that the Rous cells are not autonomously growing cells but have a limited life span. A continued release of virus 'converting' normal cells into Rous cells may be necessary for sustained progressive growth.

Neither the rate of growth, the histological appearance, nor the virus content of the tumours that grew progressively until the death of the animals or of the tumours that eventually regressed differed significantly until some 25 days after virus inoculation. At that time the tumour seemed to reach a 'turning point' after which it either continued to progress or regressed. The factors that determined the fate of the tumour are unknown but seemed to be of host origin. This is concluded from the concentration of all regressing tumours to a few birds and from the observation that no bird carried a progressing and a regressing tumour simultaneously.

#### SUMMARY

A dilution of Rous sarcoma virus was inoculated into both wings of 36 chickens. The volumes of the ensuing tumours were estimated from repeated measurements of the diameters. Serial biopsies from the tumours were assayed for virus and examined histologically.

The material was divided into two main groups: progressive lethal tumours and regressing tumours. No significant differences with respect to growth rate, content of virus and histology was found between progressively growing tumours that later regressed and those which continued to progress. Tumours in a regressing phase showed

The findings are compatible with the hypothesis that sustained progressive growth of Rous tumours is normally only possible if normal cells are continuously converted into Rous cells by released virus.

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In preparation

## STUDIES ON ODONTOGENIC CYST EPITHELIUM

### 2 Clinical and Roentgenologic Aspects of Odontogenic Keratocysts

By

J J PINDBORG and JES HANSEN

Received 23 xii 62

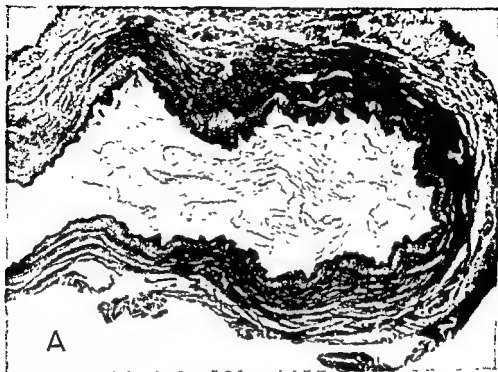
Keratinization of the epithelial lining sometimes occurs in cysts of the jaw. Over the last 40 years case reports have sporadically appeared describing such keratinization in follicular, radicular and residual cysts. Only recently have larger materials been utilized to study the frequency pattern and general characteristics of odontogenic keratocysts.

Philipsen 1956 in a review of available literature suggests the term odontogenic keratocysts for all odontogenic cysts regardless of type showing keratinization of the epithelium. This terminology would include keratinized cysts described variously as epidermoid cysts of the jaws (Broesh 1938), jaw cholesteatomas (Hauer 1926) (including cysts showing cholesterol splots (Roussy 1912, Rodier 1920)), multiple cysts of the jaws (Schleisslein 1954), and *dysembryoplastes epidermiques des maxillaires* (Dechaume et al 1958).

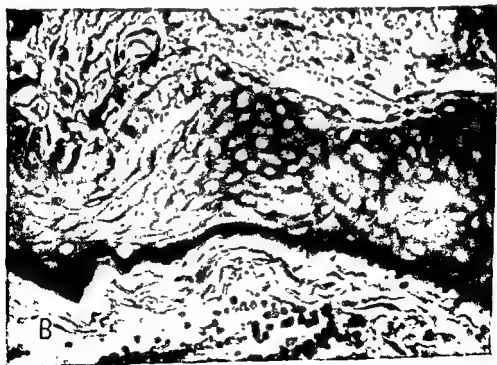
The frequency of odontogenic keratocysts found seems to vary with the type of cyst investigated, and the frequencies reported by various authors therefore reflect the make up of their study material. Langer (1947) observed a 4.2 per cent keratinization among radicular cysts, Gorlin (1957) 32 per cent among 200 follicular cysts, Dechaume et al (1958) 3.3 per cent among 300 cystic lesions of the jaws.

The present authors (Pindborg et al 1962) reviewing the 1952-1960 records of the Department of Oral Pathology, found 26 (3.3 per cent) keratinized cysts among 791 odontogenic cysts recorded. A 17 per cent keratinization was found among residual cysts, 1 per cent among radicular cysts and 7 per cent among follicular cysts. Details of the total study material and results of the histological examinations including PAS staining were reported in detail in the first paper.

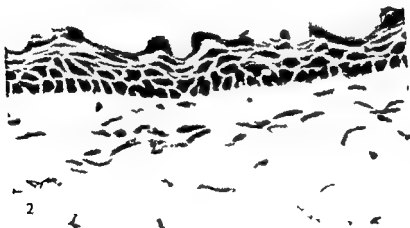
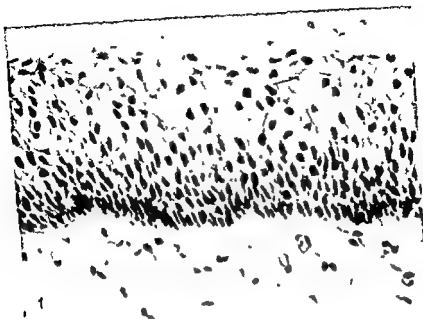
The frequency of these cysts, their roentgenographic similarity to ameloblastomas and other tumors, their liability to recur and the significance of keratinization would seem to warrant a more thorough discussion of their clinical and roentgenographic aspects, which is given here.



A Mallory-stained odontogenic keratocyst with thin epithelium and lumen filled with keratin flakes.  $\times 88$ .



B Mallory-stained odontogenic keratocyst. The parakeratotic layer is red. Rete pegs are missing.  $\times 360$ .



*Fig 1*

Epithelium from odontogenic keratocyst. Note columnar shape of basal cells  $\times 40$ .

*Fig 2*

Epithelium from odontogenic keratocyst. The basal cell layer is reduced in thickness  $\times 600$ .



Fig 3

Epithelium from odontogenic keratocyst. Note vacuolization of cells in basal cell layer  $\times 860$

### *Microscopic Characteristics of Odontogenic Keratocysts*

The epithelium has a characteristic appearance on histological examination. Frequently it is very thin, consisting of 5 or 8 rows of cells, and shows no rete pegs, Colour Plate A. The superficial layer of epithelium, *i.e.* the layer toward the lumen of the cyst, is often wavy. The basal cell layer is well defined and is composed of either columnar or cuboidal cells, Fig 1. The stratum spinosum is very scanty (Figs 1 and 2), and sometimes a direct transition of cells from the basal to the superficial layer is observed. When a stratum spinosum is present, many of the cells are vacuolated, Fig 3.

The keratinization of the epithelium is predominantly of the para-keratotic type. It is clearly demonstrated with Mallory's connective tissue stain (Colour Plate B) where the keratinized layers stain bright red against the underlying blue connective tissue cells. Despite the

thinness of the epithelium the production of keratin can be extensive. The cyst lumen may be partly occluded by keratin flakes. Colour Plate A

### MATERIAL OF PRESENT INVESTIGATION

Thirty odontogenic keratocysts removed from 27 patients were studied. Most of the cysts were also included in the material reported in the first paper Pindborg *et al.* 1962.

The patient group comprised an equal number of males (14) and females (13) in an age group ranging from 15 to 45 years.

### FINDINGS

#### *Type and location*

Distribution of cysts by type and location is shown in Table 1. Seventeen (57 per cent) of the keratocysts were typed as residual cysts (Fig. 4). A residual cyst is defined as an odontogenic cyst persisting in the jaw after removal of the involved tooth. Seven (23 per cent) were follicular (dentigerous) cysts (Fig. 5), whereas only 1 was diagnosed as a doubtful radicular cyst. Four cysts (13 per cent) were found in relation to vital teeth without any apparent connection with the teeth, Figs. 6 and 7. The location of these cysts in between or apical to normally erupted teeth seems it unlikely that the cysts were derived from primary teeth. One cyst (3 per cent) was classified as a globulo maxillary cyst.

TABLE 1  
*Type and Location of 30 Keratocysts in the Jaws*

Type	Location		
	Mandible	Maxilla	Total
Residual	14	3	17
Follicular	7	2	9
Radicular (?)	1	1	2
Globulo maxillary		1	1
Unknown origin*	4		4
Total	26	7	33

\* In relation to vital teeth

Of the 30 cysts 23 (77 per cent) were located in the mandible, 16 of them in the area of the third molar or the ascending ramus. 7 were located in the incisor, canine and premolar areas of the maxilla. Multiple keratocysts were found in two patients, three cysts in one and two in another.

#### *Röntgenography*

The small keratocysts often resembled non-keratinized cysts on roentgenographic examination (Fig. 7), whereas the larger cysts often presented a characteristic picture. Whereas the outer limits of the larger



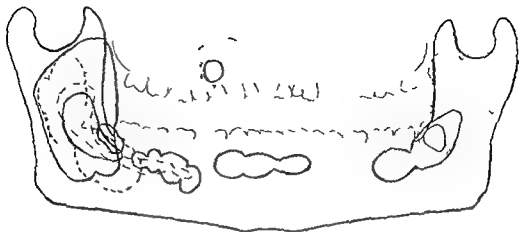


Fig 4

Schematic drawing of 17 odontogenic keratocysts originated in residual cysts



Fig 5

Schematic drawing of 7 odontogenic keratocysts originated in follicular cysts

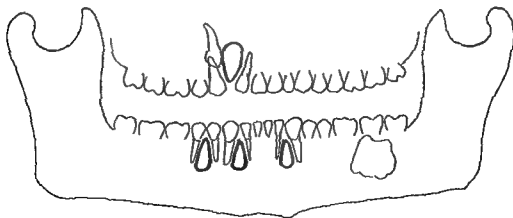


Fig 6

Schematic drawing of 4 keratocysts of unknown origin

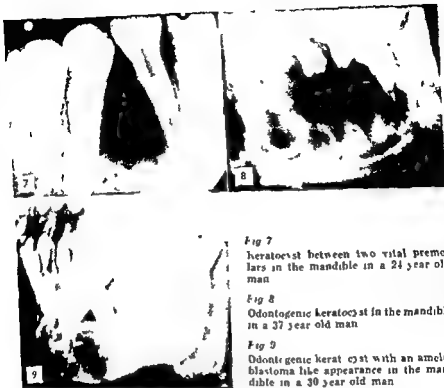


Fig 7

Keratocyst between two vital premolars in the mandible in a 24 year old man

Fig 8

Odontogenic keratocyst in the mandible in a 37 year old man

Fig 9

Odontogenic keratocyst with an ameloblastoma like appearance in the mandible in a 30 year old man

cysts normally were outlined by a narrow, radiopaque structure (Fig 8) some cysts exhibited small, rounded festoons at the periphery, Fig 11 B Moreover the large cysts often appeared multilocular, resembling the structure seen in ameloblastomas and myxomas Fig 9 Strikingly many cysts both in the maxilla and mandible were extremely large, Fig 10 A and B

### Macroscopic Findings

During operation the cyst lining was often found of a thinness which made it difficult to obtain enough tissue for histological examination The content of the cyst cavity was often paste like and of a yellowish-white colour This material consisted of desquamated keratin flakes

### Recurrence

An attempt was made to evaluate recurrence in the present material Each of the 27 patients was asked to return for re examination 14 patients returned for re examination The remaining 13 includes both those who did not return for re examination and those who were lost to follow-up. Of the 14 patients who returned for re examination 10 were reported upon with no recurrence and 4 patients presented a total of 10 recurrences. Of the 13 patients who did not return for re examination 10 were reported upon with no recurrence and 3 patients presented a total of 3 recurrences. Because of the recurrences the total



Fig 10

A and B Large odontogenic keratocyst in the ramus in a 20-year old woman

amount of operations (original operations + operations for recurrences) was 26. Among these 26 operations 21 were performed more than six months ago. 13 of these operations were followed by a recurrence. It means that whether the determination of recurrence rate is based upon the number of cysts (10 out of 16, 62.5 per cent) or the number of operations (13 out of 21, 61.9 per cent) the result is the same: 62 per cent recurrences.

In 13 cases the cyst was totally extirpated and in 7 cases a fenestration was performed. In one case the method of operation could not be determined. There was no correlation between the size or location of the cyst and its tendency to recur, nor was any difference in recurrence observed for the two surgical procedures employed.

#### DISCUSSION

The type and location of keratinized cysts reported in this paper are in general agreement with findings reported by other authors (Stein 1960, Heidsteck 1953, see also Philipsen 1956) and taken as a whole may throw some light on the aetiology of the condition. 57 per cent of the keratocysts in our material developed in residual cysts, largely in the mandible. Probably most of these cysts have developed from the enamel organ of a molar and have been left when the tooth was ex-



Fig 11

- A Lateral radiograph of a recurrent odontogenic keratocyst in the ramus of a 50 year old man  
 B Laminagram of the same area shown in A Note the festooned margins of the keratocyst

tracted, or in the angle and ramus of the mandible, have grown from distal extensions of the dental lamina, *Heidsteck 1953, Offenbauer 1962*. After extraction of the tooth the cysts evidently continue to grow in a quiescent way and often for a long time, in any event they have usually reached a good size when they are diagnosed, cf Figs 4 and 5. 13 per cent developed in cysts located in between the roots of vital teeth (Figs 6 and 7), and it might be supposed that these cysts have also developed from remnants of the dental lamina, the so called epithelial pearls of Serres. In brief, there seems to be a tendency for keratocysts to develop in cysts with slight or no inflammation of the cyst wall. Radicular cysts, developing from inflamed granulomas, rarely become keratocysts in contrast to follicular cysts which often do so. An indication of a developmental disturbance may be seen in the occasional occurrence of sebaceous glands in the cyst wall *Dechaume et al 1958*.

*Shear 1960* suggests that all cysts found in the area of the lower third molar and/or edentulous mandible should be regarded as primordial cysts. This postulate may not be justified in view of the large proportion of mandibular residual cysts reported here and elsewhere. It is probable that most such cysts actually have developed from remnants of dental structures.



Fig 12

Transition of unkeratinized epithelium into parakeratotic epithelium in an odontogenic keratocyst. The transition occurs when inflammation disappears  $\times 107$

Other peculiarities of odontogenic keratocysts become evident from a study of the literature. A number of cases are characterized by being multiple, *Offenhauer* 1962, *Dechaume et al* 1958, *Radden & Janes* 1956, *Catania* 1952, *Schlefsstein* 1943, *Caldwell & Thompson* 1955. Both multiple and solitary odontogenic keratocysts may exhibit a familial occurrence, as reported, among others, by *Thoma & Blumenthal* 1946, *Einfeldt* 1961. *Gross* 1953 and *Gorlin & Goltz* 1960 have both described odontogenic keratocysts in patients with multiple nevoid basal-cell epithelioma and bifid ribs with a familial background. The material thus far published is still of a case-report nature but indicates that certain individuals may have a hereditary predisposition for a formation of odontogenic keratocysts. This predisposition may also include a tendency to develop trichoepitheliomas or basal cell epitheliomas. The formation of keratin in cysts as well as in skin tumors is clearly illustrated by *McKelvey et al* 1960. It might well be of value to study more closely the large and accumulating material at hand on patients developing odontogenic keratocysts.

The recurrence of odontogenic keratocysts has been emphasized by several investigators (*Catania* 1952, *Heidsteck* 1953, *Einfeldt* 1961, *Offenhauer* 1962) and is striking when compared to the relative rarity of recurring non-keratinized cysts. The recurrence rate may be even higher than now supposed as recurring cysts are difficult to detect in

ordinary radiographs Fig 11 A and B illustrates a recurrent odontogenic keratocyst diagnosed by the use of laminography. Why keratinized cysts should be more apt to recur than non-keratinized cysts is obscure. Serial section of a number of the cysts in this investigation did not show any epithelial proliferation, which could be a possible explanation of a tendency to recur.

Some practical clinical consequences of the present findings deserve emphasis.

- 1) Roentgenologic examination of odontogenic keratocysts often shows changes that may simulate the presence of a tumor, especially an ameloblastoma. The literature shows that an incorrect diagnosis (roentgenologically and histologically) of an ameloblastoma for an odontogenic keratocyst has led to resection of the jaw, Einfeldt 1961. A properly evaluated biopsy should be a *conditio sine qua non* in cases of suspected tumor.
- 2) As re-examination has revealed a considerable recurrence of odontogenic keratocysts it is very important a) to secure a complete removal of the cyst wall and b) to keep patients under close observation for at least 1-2 years after the operation.
- 3) It is not always possible to make the diagnosis of a keratocyst on clinical and roentgenographic data alone. Therefore, all tissue removed from cysts should be subjected to histological examination.

## SUMMARY

Clinical and roentgenologic findings in 30 odontogenic keratocysts are reported. The majority of these cysts are found in residual cysts, next in frequency are follicular cysts. A special group comprises cysts in the mandible in relation to erupted vital teeth. The roentgenologic appearance is often characterized by festooning margins and a multilocular structure. By re-examination of 14 patients and 21 operations it was found that 62 per cent of the odontogenic keratocysts had recurred. The practical consequences of these findings are discussed.

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## DILATED RENAL COLLECTING DUCTS IN A NEWBORN INFANT AS A PRECURSOR TO MEDULLARY SPONGE KIDNEY

*Report of a Case*

*By*

ILMARI LINDGREN

Received 1963

The medullary sponge kidney is considered to be a congenital malformation. However, only 2 of the 44 patients with medullary sponge kidney were detected before the age 20 in the series of *Ekstrom et al* (1959). There are only two authors who mention the lesion in children under the age of one year (*Lenarduzzi* 1952, *Cabane* 1957). Particularly from the aetiological point of view the registration of the early postnatal cases are of importance and in the following a report of a boy aged 4 days with bilateral dilated renal collecting ducts is given. These lesions are thought to demonstrate an early phase of medullary sponge kidney.

### CASE REPORT

The case was a male infant who was the third child of a healthy

13061





*Fig. 1*

Low power view of part of a renal lobe. The medulla is composed of dilated collecting ducts  $\times 30$

normal and there were no ducts of normal size at the papillary tips. The duct became wider towards the papillary tip where their diameter was approximately 0.3 millimetres (Fig. 1).

The enlarged ducts contained some cellular debris, which showed a moderate positive PAS-reaction. The ducts were never totally filled with the material, which however increased in amount towards the papillary tip. The epithelial lining of the ducts consisted of cuboidal cells which showed a tendency to flatten the nearer they were located to the papillary tip (Fig. 2). There were no signs of inflammation in the kidney tissue. To study the possible mineral deposits in the formalin fixed renal tissue, ultrasoft microradiography was used at wavelength 8.20 Å (Engström

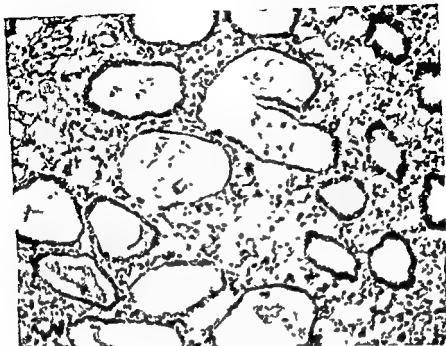


Fig 2

Detail of the center of the medulla showing the dilated ducts with some cellular debris in the lumina  $\times 200$

1966) Apart from the presence of small deposits of urates in the cortico-medullary zone no mineral salts were found and the material in the lumina of the ducts had a lower x ray absorption than the soft tissue in general (Fig 3)

In the absence of other changes in the organs examined at autopsy the renal changes were considered the primary cause of death

#### COMMENT

In their series of 15 histologically examined cases of medullary sponge kidney *Fästrom et al* (1959) did not find any changes of a type indicating transition to congenital polycystic kidney. As the morphological description of early uncomplicated cases of medullary sponge kidney is still lacking no absolute evidence can be obtained if the reported case should have developed to a sponge kidney with large medullary cysts. However in this case there were no cysts in the cortex and the lesion clearly differs from the renal dysplasia usually encountered in children (*Ericsson & Ivemark* 1958 *Parkkulainen et al* 1969) this case can be considered as an early phase of medullary sponge kidney. The cause of death in this particular case was apparently a direct result of the renal changes.

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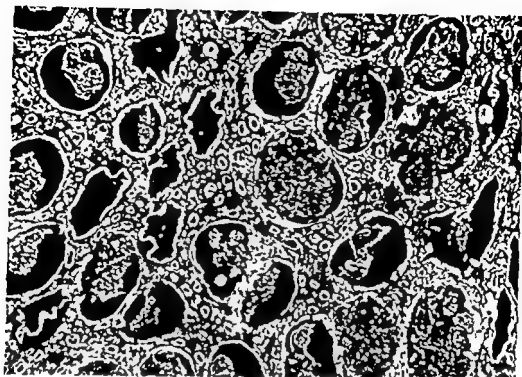


Fig 3

Microradiogram of a 5 micra thick section from the lower part of the medulla. Exposed at 8-20 k. The material in the lumina has a lower x-ray absorption than the epithelial lining of the ducts and the interstitial tissue. There are no mineral salt deposits.  $\times 180$

x-ray-absorbent substances in the ducts, and the absence of inflammatory changes. These lesions, listed above and lacking in the reported case, should be considered as secondary ones and not typical for the early stage of disease.

### SUMMARY

The dilated collecting ducts in both kidneys were observed in a male infant aged 4 days at necropsy. These lesions were interpreted as an early phase of the medullary sponge kidney described in adults. The finding of the lesions in early postnatal life favours the idea that the medullary sponge kidney is due to a congenital dilatation of the collecting ducts.

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## INTRACEREBRAL, POSSIBLY MALIGNANT OSTEOCHONDROFIBROMA IN A CHILD

By

G FLYGER H FREIDENFELDT and S R ORELL

Received 13 VII 62

Primary intracranial tumours with a chondromatous or osteochondromatous structure are very rare (11). The number of such cases described in the literature is at present about 40, principally males between the ages of 18 and 65. Tumours of this type sometimes originate in the base of the skull but most of them are in no way connected with the bones of the cranium. They are generally attached to the dura mater or the falx and localized to the convexity (2, 3, 4). In certain cases it has not been possible to find any connection between the tumour and the meninges (2, 6). Two cases of intraventricularly situated tumours have also been described (7).

The majority of these tumours are highly differentiated, well encapsulated chondromata with a structure similar to benign chondromata elsewhere in the body (2). Osteochondromata are less common (1, 8, 12). Besides cartilage and bone tissue of varying differentiation certain tumours also contain cellular parts with a fibromatous or meningiomatous structure (5). The tumours are often calcified and are thus visible on plain skull roentgenograms. In a few cases, on account of high cellularity, polymorphism and diffuse infiltration into the surrounding tissue the tumours have been regarded as malignant, a fact which has been verified by the clinical course (1, 3, 10, 13).

The clinical picture is similar to that of any intracranial tumour correspondingly located though of course it varies according to the speed of growth. Growth can be extremely slow in the case of a well differentiated chondroma and much swifter in a tumour which is principally of a fibromatous, cellular nature.

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to all from embryonal cartilaginous rests in the cranial bones (2). The tumours attached to the meninges are believed by certain authors to originate from embryonal cartilaginous rests in the dura displaced from the base of the skull (5, 8, 9, 12). Other authors claim that they originate from meningeal fibroblasts or

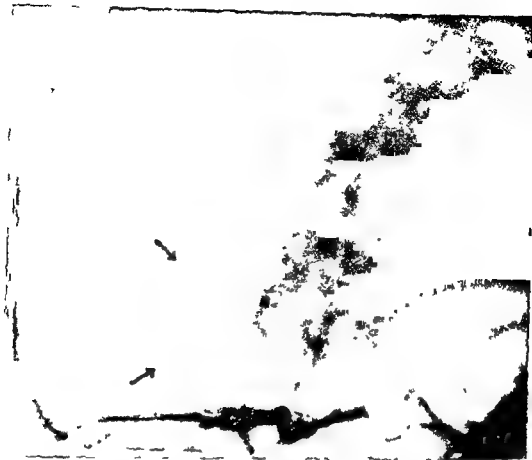


Fig 1a

Plain skull radiogram showing multiple nodular (x) and a linear (x) calcification in the subfrontal region. Widening of coronary sutures.

multipotent mesenchymal cells in the *dura* or the *arachnoidea* (1, 2, 10). A direct transition from typical meningiomatous tissue to chondromatous tissue has been described, as by *Cushing* (3, 5). Even in the rare cases where there is no visible connection between the tumour and the meninges a connection with the *arachnoidea* has been assumed (2). The intraventricular tumours are thought to arise from the *choroidal plexus*.

#### CASE REPORT

11 years old, previously healthy and without known trauma of the head. Examination at another hospital revealed that the patient's somatic and neurological status was normal apart from a plain skull roentgenography revealed considerable right frontal lobe (Fig 1a). The patient died on 12 1961.



Fig 1b

Catheter angiography, early arterial phase. Backward upward displacement of the anterior cerebral and anterior communicating arteries and of the inferior part of the pericallosal artery. The subfrontal vessels are stretched.

The subfrontal segment of the tumour contained numerous small calcifications and a tiny linear calcification in its upper posterior border (Fig 1). Encephalography was not performed.

Operation was performed in 1962 under general anaesthesia with controlled

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Fig 1a

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Fig 1c

Carotid angiography late arterial phase. Irregular pathologic vessels. Early filling of a large irregular drainage vein.

### PATHOLOGY

The tumour removed was rounded and measured  $5 \times 4 \times 3\frac{1}{2}$  cm. It was solid with a firm, almost hard consistence. The central part seemed to contain cartilage and bone and was to a great extent calcified while the peripheral parts were softer with a grey white striated cross section. The tumour was surrounded by a distinct capsule which however had been somewhat damaged by the operation. On the capsule surface there were numerous vessels.

Histological examination revealed that a large part of the tumour consisted of highly cellular mesenchymal tissue made up of medium sized spindle shaped cells. The nuclei were rather large, oval or elongated, sometimes vesicular and moderately polymorphous. Here and there were a number of mitotic figures. The tumour cells were arranged in interwoven bundles but showed no tendency to form whorls or palisading of the nuclei. The cellular areas contained very little collagenous intermediary substance. Silver impregnation however revealed a fairly manyfold reticulin network. There were no psammoma bodies.

The central parts of the tumour contained plenty atypical, partly calcified osteoid and cellular immature cartilaginous tissue which was also partly calcified. The transition from cellular fibromatous tissue to cartilage and osteoid was indistinct. In this area the tumour thus was very reminiscent of an osteogenic sarcoma. No giant cells were observed. The tumour was surrounded by a thin connective tissue capsule and showed no tendency to invade this in the sections examined (Fig 2).

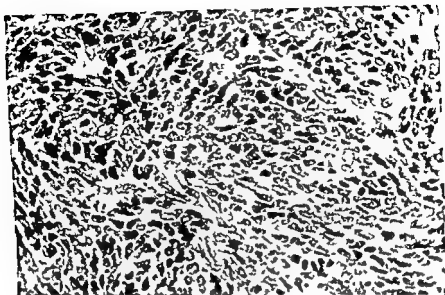


Fig 2a

Highly cellular fibromatous area showing moderate polymorphism  
Hematoxylin eosin  $\times 370$

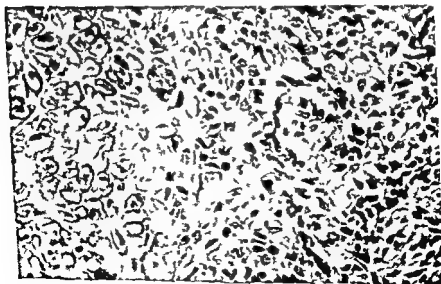


Fig 2b

Showing chondromatous and fibromatous tissue in between atypical osteoid  
Hematoxylin eosin  $\times 350$



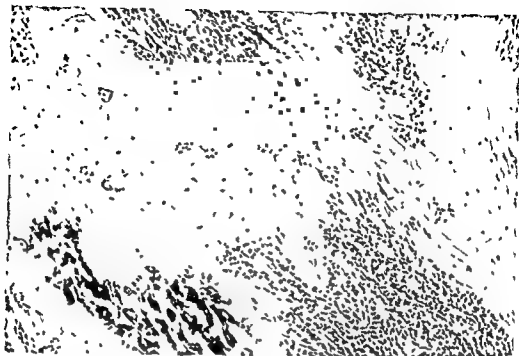


Fig 2c

Cellular chondromatous and osteoid tissue with calcification van Gieson  $\times 100$

### DISCUSSION

The discovery of a cellular, intracerebrally situated mesenchymal tumour with osteochondromatous components first indicated a metastasis, probably from a primary bone tumour. Roentgenographic examination of the entire skeleton and the lungs, however, revealed no pathological changes. On account of the roentgenographical picture and the observations made during the operation, the possibility of an osteogenic sarcoma originating from the base of the skull was also ruled out.

It was thus a question of a primary intracranial tumour of the same nature as the group of intracranial chondromatous tumours described at the beginning. The patient was remarkably young, 11 years old. So far as we can ascertain there is only one other known case of a tumour of this type in a child. Wolf & Echlin gave a report of a 9-year-old girl who was operated for an osteochondrosarcoma which was attached to the falx and which infiltrated into both of the frontal lobes. The patient died shortly after the operation (13). In our case the tumour was highly cellular and in places fairly rich in mitoses and containing atypical osteoid. Histologically it was therefore of a malignant character. Judging from the short history, furthermore, it must have grown quickly. On the other hand even microscopically the tumour was well defined and easy to remove at the operation. Naturally, the period of observation, six months, is still too short to permit a definite prognosis.

This case is particularly interesting inasmuch as the entire tumour was unquestionably deeply intracerebrally situated and in no way connected either with the falx, the leptomeninges or the base of the skull. Nor did histological examination show a structure which could be characterized as meningiomatous. Naturally, it is difficult to establish the pathogenesis of the tumour with any certainty. To us it seems most likely that it originated from displaced embryonal cell rests of a mesenchymal nature. The age of the patient gives a certain support to this theory.

#### SUMMARY

A case is described of an intracerebral tumour with an osteochondrofibromatous structure in an 11-year old boy. Histologically the tumour was of a malignant character, highly cellular and containing atypical osteoid, though it was well defined and easy to remove during the operation. Six months after the operation the patient was completely free of symptoms. Particularly interesting from the pathogenetic point of view was the fact that the tumour was deeply intracerebrally situated without being connected to the dura, the falx or the base of the skull.

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#### ADDENDUM

In June 1963, 17 months after the operation, the patient reported that he was going to school and was perfectly well and free from symptoms.

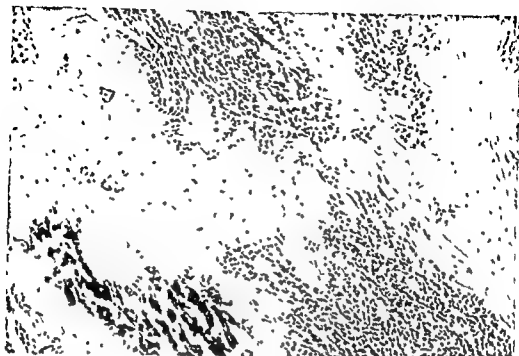


Fig 2c

Cellular chondromatous and osteoid tissue with calcification van Gieson  $\times 100$

### DISCUSSION

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referring to those tumours which are diagnosed in literature as atypical seminoma (Collins & Schoenenberger 1962), unusual dysgerminomas or embryonal carcinoma with gonadoblastomatous part (Santesson & Marrubini 1957). These questions are important and of great interest but they must be discussed elsewhere.

Now I will discuss separately the feminizing form of mixed germ cell tumour (gonocytoma II) and the masculinizing form (gonocytoma III or gonadoblastoma). In this paper I will try to present both pathological and endocrinological aspects.

### A FEMINIZING FORM OF MIXED GERM CELL TUMOUR (GONOCYTOMA II)

This tumour is built of germ cells and Sertoli granulosa cells. From six previously described cases we can accept the following clinicopathological features of Gonocytoma II (2a). The tumour occurs in children and adolescent girls. Sex chromatin pattern is positive in accordance with the female character of external genitalia. The puberty may either be precocious or normal. Menarche may frequently be premature and followed by irregular uterine bleeding. No congenital malformations are observed which frequently are found in gonadoblastoma (Gonocytoma III). From the endocrinological viewpoint such tumours are capable of estrogen production resulting in feminization of the patient. Most probably Sertoli granulosa cells are responsible for such a hormonal situation.

Perhaps the most remarkable case was the one I recently observed (91, 2a) and which I consider to be typical for feminizing mixed germ cell tumour. An 8 year old girl was examined because of symptoms of precocious puberty. First vaginal bleeding occurred when she was six years old. Bleeding episodes from then on occurred every two to three months. Symptoms of premature feminization including womanly stature and fatty tissue topography, premature development of breasts and pubic hair growth was noted. Gynecologic examination revealed marked development and pigmentation of the labia minora.

At laparotomy the ovaries showed proliferative endometrium. A year later she was operated on and on the right side an ovarian tumour was found 8 cm in diameter.

Histological examination showed lobulated neoplastic tissue composed of cells arranged in round nests separated by partially hyalinized bands of connective tissue (Fig. 1). Two types of cells could be recognized: 1) germ cells and 2) Sertoli granulosa cells.

## THE MIXED GERM CELL TUMOURS WITH HORMONAL ACTIVITY

By

JERZY TETTER

Received 11.1.63

The mixed germ cell tumours have been recently reported with an increasing frequency (1, 2, 9, 12, 14, 15, 16, 17, 18, 19, 22, 25, 26). The remarkable feature of such tumours is that they are capable of sex hormone production, their hormonal activity may be feminizing or masculinizing.

At the outset I would like to briefly recall my classification of gonocytomas, proposed in 1960. Since such tumours originate from gonocytes the term "Gonocytoma" suggested by Teitum in 1946, seems to be most appropriate. They were divided into four groups (23).

Gonocytoma I—a homogenous form of tumour formed solely of germ cells (dyserminoma—seminoma). They are hormonally inactive (20, 21).

Gonocytoma II—a mixed form consisting of two types of cells occurring in the embryonic gonad—germ cells and Sertoli granulosa type cells. These tumours have feminizing properties.

Gonocytoma III—corresponds to Scully's gonodoblastoma and is built of germ cells, Sertoli granulosa cells and interstitial Leydig cells. Calcified concretions in the tumour parenchyma are a conspicuous feature of this class. This group of tumours has masculinizing properties.

Gonocytoma IV—homogenous form of gonocytoma with a marginal proliferation of androgenic interstitial cells (analogous to the theca-lutein reaction seen in the ovarian stroma surrounding metastatic nests of Krukenberg's tumours).

The possibility of pre-operative diagnosis in such tumours as well as the problem of differentiation between feminizing and masculinizing forms are the subject of this lecture.

I would also like to note, that certain rare neoplasms occur which most probably belong to mixed germ cell tumours but whose very complex cyto-architecture makes the exact diagnosis impossible. I am

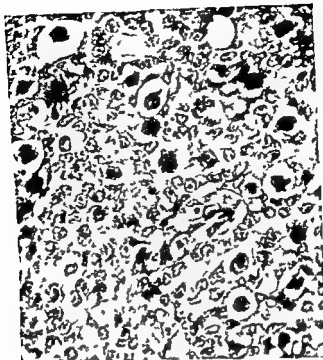


Fig 1b

Feminizing form of mixed germ cell tumour (Gonocytoma II)

Area of a confusing admixture of germ cells and sex cord cells in malignant fashion

aged 15 operated on in our Clinic in 1938. The other one observed by Dr Hughesdon from London of a 16 year old girl with symptoms of metrorrhagia. Both were described in my previous report (20) together with three cases from pre war literature. It is notable that all cases of gonocytoma II collected from literature occurred in children and adolescent girls (23). Uniformity of clinical and pathological features of the described type of tumours enables their classification as a definite clinico-pathological entity: feminizing mixed germ cell tumour (Gonocytoma II).

#### A MASCULINIZING FORM OF MIXED GERM CELL TUMOUR (GONOCYTOMA III)

This kind of tumours was originally described by Scully in 1903 and given the name of gonadoblastoma since it was found to recapitulate the embryonic development of the gonad better than does the pure gonocytoma.

The neoplasm is composed of germ cells, Sertoli granulosa and interstitial Leydig cells. Its conspicuous feature is the presence of calcified concretions in the parenchyma. The tumour occurs in patients with



Fig 1 a

Feminizing form of mixed germ cell tumour (Gonocytoma II)

Single germ-cells scattered inside the nest are most closely related to the Sertoli than to the granulosa (arrow) type. In the other region sex-cord cells differentiate toward granulosa cells and form a microfollicular pattern. Connective tissue bands separate the neoplastic nests and contain only a few spindle shaped cells.

losa cells and in some places were encircled by them. Small areas of the tumour were characterized by a confusing mixture of germ-cells and sex-cord cells in malignant fashion (Fig 1 B). Connective tissue which separated neoplastic nests contained only few spindle-shaped cells. The stroma did not contain any Leydig cells. Unlike cases of embryonal tumours of testicular type (Scully's gonadoblastoma or Melicow's gonadoma) calcified concretions and clumps of Leydig cells are never found here.

The histological investigation showed that in certain areas the sex-cord cells were more closely related to the Sertoli than to the granulosa type of cells (Fig 1 A). It is known that in human beings Sertoli-cell tumours of both testis and ovary are feminizing (Teilum 1958, Morris & Scully 1958). On the other hand microscopic studies also disclosed that sex-cord cells differentiated in some regions toward granulosa cells and formed a microfollicular pattern (Fig 1 A) resembling folliculoma. Such tumours usually produce also feminizing effects.

Two similar cases of Gonocytoma II may be quoted. One is of a girl



Fig. 2c

Masculinizing form of mixed germ cell tumour (Gonocytoma III Gonadoblastoma)  
 Microscopic picture of a section from the tumour of the right gonad. Typical foci  
 of calcification and small nest of neoplastic cells.

somatic sexual disturbances (eunuchoidal features or signs of Turner's syndrome) reared as women and having female type of external genitalia but with symptoms of masculinization. In all reported cases of gonadoblastoma negative (male) sex chromatin pattern was found (see Table 1). From the endocrinological viewpoint such tumours are capable of sex hormone production, mainly of androgens.

Recently we reported a new case of gonadoblastoma (26) which I consider most illustrative for the masculinizing group of mixed germ cell tumour. A patient aged 21, reared as a woman, was examined because of primary amenorrhea, retardation of sexual development and virilism. Two uterine bleeding episodes occurred when she was 20. She was a mainly statured female with eunuchoidal body proportions. Acne, seborrhea, slight temporal baldness were present as well as signs of facial and body hirsutism. The breasts were of pubertal type. The clitoris was hypertrophied, the vagina well formed, the uterus was small.





Fig 2a

Masculinizing form of mixed germ cell tumour (Goncoestroma III Gonadoblastoma)  
Hysterosalpingogram. The gonadal tumour contained faint floccular foci of calcification. Small uterine cavity and patent fallopian tubes.

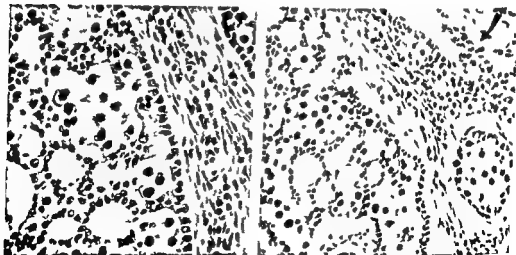


Fig 2b

Masculinizing form of mixed germ cell tumour (Goncoestroma III Gonadoblastoma)  
Microscopic findings in a section from left dysgenetic gonad showing typical gonadoblastoma pattern. Note the two large nests composed of Sertoli granulosa cells. The former are the small cells with oval carrot shaped nuclei arranged in a single row along the periphery of the nest which form a folliculoid pattern or encircle the germ cells. The latter have large nuclei and clear cytoplasm. Between the nests small clumps of immature Leydig cells are seen.

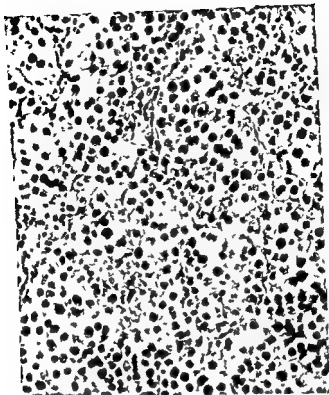


Fig 2 c

Masculinizing form of mixed germ cell tumour (Gonocytoma III Gonadoblastoma)  
A large nest of germ cells similar to "pure dysgerminoma"

on the right, behind the uterus. It contained faint floccular foci of calcifications (Fig 2 A). This finding with all the above evidence, enabled us to diagnose this tumour, before the operation—as a gonadoblastoma.

Laparotomy disclosed an infantile uterus and fallopian tubes. On the left a typical dysgenetic, whitish streak in the place normally occupied by the ovary, and on the right a gonadal tumour were found. The tumour measured 5 by 3 by 2.5 cm, was covered by a yellowish gray, smooth glistening capsule and contained chondroid like nodules. On the fourth postoperative day uterine bleeding occurred. The histological examinations revealed that the cortical part of the left dysgenetic gonad built of scanty connective tissue, resembling ovarian stroma. In the medullary part a small nodule was found, and recognized as incipient gonadoblastoma. The nodules contained germ cells scattered in three types:

1) in three types  
2) in peripheral single file along the base of the



Fig 2d

Vasculinizing form of mixed germ cell tumour (Gonocystoma III (Gonadoblastoma)  
Calcified concretions and groups of Leydig cells

and infantile. Sex chromatin pattern was negative (male). Urinary 17-ketosteroid output was within normal limits (6.7–10.1 mg/24 hrs). Slight estrogen activity was found both in the urine (23 mcg by Jayle's chemical method) and in vaginal smears. Gonadotropin level was increased to 66 and 71 mU (Kleinfeller-Dekanski method). Human chorionic gonadotropin stimulation with simultaneous inhibition of adrenal androgenic activity by prednisone, provoked a significant increase in estrogen excretion from 23 to 47.72 mcg. The 17-KS output was only slightly affected (6.9–8.8 mg/24 hrs). This response resembled the result of HCG stimulation observed in testicular dysgenesis (3, 5, 8, 9, 10, 11). The possibility of the presence in this case of testicular dysgenesis was supported by the evidence of negative sex chromatin pattern and symptoms of intersexuality with some masculinizing features.

Radiological examinations were decisive for the diagnosis. Hysterosalpingogram demonstrated a small uterine cavity with patent fallopian tubes (Fig 2A).

The most striking and surprising disclosure was a tumour situated

References	Scully cases 1953		Her 1948	Prybylora 1960	Sulzemann 1961	Author's cases 1962	
	No. 1	No. 2				No. 1	No. 2
No. of case	1	2	3	4	5	6	7
Age	8	19	25	23	13.5	19	21
Habitus	girl	and "girls"	cunuchoidal and androidal	cunuchoidal and androidal	androidal	Turner and "boyish"	cunuchoidal and androidal
Uterus and Fallopian tubes	?	+	+	+	+	+	+
(ross pathology							
a) right gonad	tumour	tumour	rudiment	tumour	testis-like tumour rudiment	tumour	tumour
b) left gonad	?	—	tumour	dysgen streak		dysgen streak	dysgen streak
<i>Microscopic findings</i>							
Characteristic nest of germ cells and sex cord cells							
<i>Leydig cells</i>							
a) clumps	+	+	+	+	+	+	+
b) adenoma-like formation	+	+	+	++	+	++	+
Calcified concretions	+	+	+	+	+	+	++
Areas of pure gonocytes (oma)	+	+	+	+	+	+	+

All patients had female legal sex female type of genitalia and negative sex chromatin pattern together with androgenic symptoms (hypertrophied clitoris and hirsutism).

\* Classified as dysgerminoma in original report (Her 1948). The sex chromatin in this case was established by examining various nuclei in the slides of the operative specimen.

§ The author is greatly indebted to Dr Scully (Boston U.S.A.), Prof Her (Tel-Aviv, Israel), Dr Siebenmann (Zürich, Switzerland) and Dr Prybylora (Poznan, Poland) for sending slides and clinical data (reprints) from their cases of gonadoblastoma and their permission to use them in my researches.

pseudotubules, 3) in folliculoid pattern (Fig 2 B) Foci of Leydig cells were clustered between the cellular nests described above

The tumour of the right gonad presented a more typical structure of gonocytoma III (gonadoblastoma, Fig 2 C) In many areas microscopic examination showed in the hyalinized stroma small groups of Leydig cells and calcified concretions in form of irregular masses or "psammoma-like" bodies (Fig 2 D) In other parts the neoplastic nests were composed solely of germ cells in an arrangement typical for "pure dysgerminoma" (gonocytoma I, Fig 2 E) In still other portions of the tumour Sertoli-granulosa cells dominated

The following symptoms showed that the tumour was capable of sex-hormone production Four weeks after the operation urinary estrogen output decreased from 23 mcg to about 5 mcg/24 hrs The urinary 17-KS were lowered from 10.1 to 5.5 mg/24 hrs The patient complained of sweats and hot flushes These symptoms disappeared after replacement therapy was instituted and cyclic uterine bleeding ensued

I collected as many clinical data as possible together with microscopic slides from seven gonadoblastoma cases, which have been reported in recent years Table 1 presents some clinical, hormonal and pathological features of these cases Endocrine signs from my two cases have been collected in detail in Table 2

The material presented proves, that there exists a uniformity of clinical and pathological features of this type of tumours which enables their classification as a definite clinico-pathological entity masculinizing mixed germ cell tumour (Gonocytoma III) Even more, it allows detection and a preoperative of gonadoblastoma may be listed as follows

- 1) Abnormal somato-sexual development in a patient reared as a woman with primary amenorrhea and some signs of masculinization
- 2) Negative (male) sex chromatin pattern, contrasting with female type of genitalia
- 3) High gonadotropin urinary titre despite clinical and biochemical signs of sex-hormone activity (analogous with cases of testicular feminization syndrome)
- 4) A significant increase in estrogen excretion after HCG stimulation (normally observed in men or in case of testicular dysgenesis (3, 4, 8, 10, 11))
- 5) Radiological evidence of a gonadal tumour containing floccular foci of calcification The usefulness of such radiological examination in detection of gonadal neoplasm in patient with congenital somato-sexual ambiguities was first shown by Melicow & Uson (1959)

The degree of masculinization seems to be dependent on the relative number of Leydig cells (Fig 2 B and 2 D) The masculinization features (enlarged clitoris and scrotal appearance of the labia) were most marked in my first case of gonadoblastoma (Table 2) The characteristic histological picture was in full accord with this clinical features In many sections foci of Leydig cells were seen in hyalinized stroma The most marked finding, in my opinion were circumscribed agglomerations of Leydig cells in the form of small adenomas, which appeared to be hyperplastic

On the other hand, however, oestrogenic symptoms were also observed (Table 1 and 2) which probably were dependent of the Sertoli-granulosa cell component In my two cases the characteristic withdrawal bleeding occurring after removal of the tumour, proved, that the gonadoblastomas are also capable of estrogenic production It should be noted however, that in these two cases estrogenic symptoms were not as marked as the androgenic features

A separate question is that of high gonadotropin urinary values in cases of gonadoblastoma The increased excretion of gonadotropins in cases of testicular tumour, as reported by *Hamburger*, was probably due to a reduction of the total amount of normal testicular tissue In cases of gonadoblastoma dysgenetic, defective gonads and tumours tissue are unable to produce enough sex hormones to suppress the release of gonadotropin by the pituitary

#### SPECIAL PATHOLOGICAL ASPECTS

##### *Testicular Dysgenesis as a Background for the Development of masculinizing Form of a Mixed Germ Cell Tumour*

Several histological features may indicate that gonadoblastoma arises in and from dysgenetic or rudimentary testes It will simplify matters if these phenomena are collected in three groups

1) *Scully's* two first gonadoblastomas presented the unusual feature of disseminated millitary calcification which in his experience does not characterize the pure dysgerminoma Similar formations in all later reported gonadoblastomas are found as psammoma like bodies or calcified flecks or irregular masses (Fig 2 C, 2 D, 3)

In the light of present investigations it can be claimed that

*lowski* 1960, case No 2 *Dominguez & Greenblatt* 1960, 1961, 1962, 1963, 1964, 1965, 1966, 1967, 1968, 1969, 1970, 1971, 1972, 1973, 1974, 1975, 1976, 1977, 1978, 1979, 1980, 1981, 1982, 1983, 1984, 1985, 1986, 1987, 1988, 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, 2025, 2026, 2027, 2028, 2029, 2030, 2031, 2032, 2033, 2034, 2035, 2036, 2037, 2038, 2039, 2040, 2041, 2042, 2043, 2044, 2045, 2046, 2047, 2048, 2049, 2050, 2051, 2052, 2053, 2054, 2055, 2056, 2057, 2058, 2059, 2060, 2061, 2062, 2063, 2064, 2065, 2066, 2067, 2068, 2069, 2070, 2071, 2072, 2073, 2074, 2075, 2076, 2077, 2078, 2079, 2080, 2081, 2082, 2083, 2084, 2085, 2086, 2087, 2088, 2089, 2090, 2091, 2092, 2093, 2094, 2095, 2096, 2097, 2098, 2099, 2100, 2101, 2102, 2103, 2104, 2105, 2106, 2107, 2108, 2109, 2110, 2111, 2112, 2113, 2114, 2115, 2116, 2117, 2118, 2119, 2120, 2121, 2122, 2123, 2124, 2125, 2126, 2127, 2128, 2129, 2130, 2131, 2132, 2133, 2134, 2135, 2136, 2137, 2138, 2139, 2140, 2141, 2142, 2143, 2144, 2145, 2146, 2147, 2148, 2149, 2150, 2151, 2152, 2153, 2154, 2155, 2156, 2157, 2158, 2159, 2160, 2161, 2162, 2163, 2164, 2165, 2166, 2167, 2168, 2169, 2170, 2171, 2172, 2173, 2174, 2175, 2176, 2177, 2178, 2179, 2180, 2181, 2182, 2183, 2184, 2185, 2186, 2187, 2188, 2189, 2190, 2191, 2192, 2193, 2194, 2195, 2196, 2197, 2198, 2199, 2200, 2201, 2202, 2203, 2204, 2205, 2206, 2207, 2208, 2209, 2210, 2211, 2212, 2213, 2214, 2215, 2216, 2217, 2218, 2219, 2220, 2221, 2222, 2223, 2224, 2225, 2226, 2227, 2228, 2229, 2230, 2231, 2232, 2233, 2234, 2235, 2236, 2237, 2238, 2239, 2240, 2241, 2242, 2243, 2244, 2245, 2246, 2247, 2248, 2249, 2250, 2251, 2252, 2253, 2254, 2255, 2256, 2257, 2258, 2259, 2260, 2261, 2262, 2263, 2264, 2265, 2266, 2267, 2268, 2269, 2270, 2271, 2272, 2273, 2274, 2275, 2276, 2277, 2278, 2279, 2280, 2281, 2282, 2283, 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# ENDOCRINOLOGIC ASPECTS OF GONADOBLASTOMA CELLULAR SOURCE OF SEX-HORMONE PRODUCTION

In clinical pictures of gonadoblastoma androgenic symptoms are always observed (see Table 1) and seem to be the dominating factor. The androgenic activity of such tumours is due to the interstitial Leydig cells component. In all slides of the seven studied cases I was able to observe interstitial Leydig-like cells, varying in number, appearance, size and arrangement. They actually contain considerable lipochrome pigment in their cytoplasm, which is characteristic of Leydig cells. I agree with Scully<sup>1</sup> that they are most probably Leydig and not theca cells. I should however add, that I never saw any typical crystalloids of Reinke in these cells, but this is almost the general rule in testicular dysgenesis.

TABLE 2  
*Endocrine Symptoms and Their Morphological Basis in two Cases of Gonadoblastoma (Gonocytoma III) Observed by the Author*

Characteristics	Case I previously published (1960)	Case II (1962)
<b>I Symptoms of endogenous oestrogen production</b>		
1 Breast development	underdeveloped pubertal type	pubertal type
2 Vaginal development Ability to have sexual intercourse	rather poor	good
3 Spontaneous uterine bleeding	none	Two episodes of bleeding at 20 years of age
4 Withdrawal bleeding after gonadectomy (removal of the gonadal tumour)	on 8th post-operative day	On 4th post-operative day
5 "Climacteric" symptoms following operation	present	present
<b>II Symptoms of endogenous androgen production</b>		
1 Enlarged clitoris	prominent	present
2 Appearance of the labia maiora	scrotal	female
3 Tendency to hirsutism	present	present
4 Skin	pale and oily	pale and oily
<b>III Characteristics of cells capable of hormone production</b>		
1 Sertoli-granulosa type cells	present	present in abundance
2 Interstitial Leydig cells	dominating in large groups	singular, scattered
a) appearance, size	medium sized polygonal	small, polygonal
b) special arrangement	areas of adenoma-like formations	small clumps

<sup>1</sup> Scully personal communication





2) In my first case of gonadoblastoma the tumour was on the right side, and on the left a dysgenetic streak was found, in which the medullary region contained rudimentary seminiferous tubules. On the other hand microscopic investigations of the tumour disclosed in some areas nests of Sertoli-type cells and germ-cells in an arrangement resembling seminiferous tubules.

3) Most interesting aspects are presented in Siebenmann's case of a male hermaphrodite with a gonadoblastoma. The tumour was discovered at the periphery of definite testicular tissue. There was no sharp dividing line between the typical testicular tissue and the gonadoblastoma. To the contrary one could observe gradual transitions from seminiferous tubules to gonadoblastoma nests.

Lastly I would like to mention the results of HCG stimulation test. The increased excretion of estrogens after HCG stimulation suggests the presence of testicular tissue in the gonadal tumour, particularly in a patient with negative sex chromatin pattern. According to Maddock & Nelson (1952), Jayle *et al* (1958), Diczfalussy (1958), Dux (1956) such an estrogen response is specific for testicular tissue.

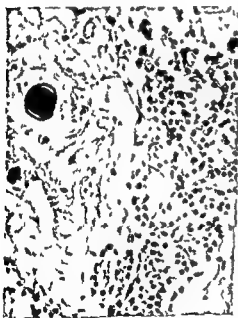


Fig 3

Fig 3 Psammoma like bodies and the calcified concretions surrounded by hyalinized connective tissue with small clumps of Leydig cell (gonocytoma III own case No 1)



Fig 4

Fig 4 Psammoma like calcification in testicular dysgenesis (case of male hermaphrodite BH aged 21 specimen No 20748)



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## RESULTS

The antistaphylolysin titre before and two weeks after treatment is given in Table 1. The same result expressed by cumulative values is illustrated in Fig. 1.

From the table and the figure, it is seen that the serum titre in the 6 animals varied initially between  $<0.36$  and  $4.0$  IU/ml of blood serum. In most of the animals the serum titre showed a considerable rise two weeks after treatment. The rise was higher, as a rule, in the 23 animals that had been treated intramuscularly than in the 43 that had been vaccinated subcutaneously. Statistically, the difference was highly significant ( $P = <0.001$ ).

## DISCUSSION

In immunization experiments with staphylococcal toxoid in general a varying rise of the antistaphylolysin titre has been noted. The variations have been attributed to differences in the vaccines used and to differences in the treated animals as producers of immune bodies. In the investigation presented here two comparable groups of animals were treated with the same vaccine, one group subcutaneously and the other intramuscularly with the purpose to detect possible differences in the immune response to the two ways of application. In both groups a rise of the antistaphylolysin titre in blood serum could be noted. The rise was significantly higher in the group treated intramuscularly.

The possibility that the result might have been influenced by a non-discovered staphylococcal infection in one group can be excluded. The animals of each group were distributed into several different herds. Moreover, a large number of control animals in each herd did not show any rise of the serum titre.

Besides, the injection of vaccine was performed in such a manner and with such care that leakage after treatment must be excluded.

Consequently, a definite relationship existed between way of application and rise of the antistaphylolysin titre in blood serum. The result inspires to continue studying immunity response for longer periods of time after subcutaneous and intramuscular treatment, respectively.

Even if the antistaphylolysin titre hardly can be interpreted as an actual indicator of an organism's state of immunity, the aim of treatment with toxoids is generally, however, to obtain the greatest possible rise of the serum titre. On the basis of the present results, intramuscular application may be recommended as preferable to subcutaneous in toxoid treatment of cattle.

## SUMMARY

Two groups of cows were treated with a combined staphylococcal vaccine. In one group the vaccine was injected subcutaneously and in

TABLE 1

*Antistaphylolysin Titre in 43 Cows Vaccinated Subcutaneously (Sc) and in 23 Cows Vaccinated Intramuscularly (Im) before and two Weeks after Treatment, Respectively*

Antistaphylo- lysin titre		<0.36	0.36	0.5	0.7	1.0	1.4	2.0	2.8	4.0	5.6	8.0	11	16	22	28	44	64	88	128
Sc	Before	7	■	■	3	9	1	7	5	-	-	-	-	-	-	-	-	-	-	-
	After	1	-	1	-	■	3	11	2	5	2	5	3	5	-	1	-	-	-	-
Im	Before	3	1	7	-	8	1	-	1	2	-	-	-	-	-	-	-	-	-	-
	After	-	-	-	-	-	-	-	1	3	2	4	1	5	2	2	2	-	-	1

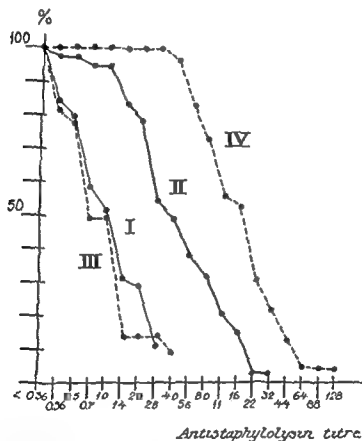


Fig 1

- Abseissa Antistaphylolysin titre (I.U.)  
 Ordinate % animals, each dot gives the percentage of animals in the group that has a corresponding or higher antistaphylolysin titre
- Curve I — Antistaphylolysin titre in 43 animals  
 Curve II — Antistaphylolysin titre in the same 43 animals two weeks after subcutaneous vaccination  
 Curve III — Antistaphylolysin titre in ■ animals  
 Curve IV — Antistaphylolysin titre in the same 23 animals two weeks after intramuscular vaccination

## RESULTS

The antistaphylolysin titre before and two weeks after treatment is given in Table 1. The same result expressed by cumulative values is illustrated in Fig. 1.

From the table and the figure, it is seen that the serum titre in the 66 animals varied initially between  $<0.36$  and  $4.0$  IU/ml of blood serum. In most of the animals the serum titre showed a considerable rise two weeks after treatment. The rise was higher, as a rule, in the 23 animals that had been treated intramuscularly than in the 43 that had been vaccinated subcutaneously. Statistically, the difference was highly significant ( $P = <0.001$ ).

## DISCUSSION

In immunization experiments with staphylococcal toxoid in general a varying rise of the antistaphylolysin titre has been noted. The variations have been attributed to differences in the vaccines used and to differences in the treated animals as producers of immune bodies. In the investigation presented here two comparable groups of animals were treated with the same vaccine, one group subcutaneously and the other intramuscularly with the purpose to detect possible differences in the immune response to the two ways of application. In both groups a rise of the antistaphylolysin titre in blood serum could be noted. The rise was significantly higher in the group treated intramuscularly.

The possibility that the result might have been influenced by a non-discovered staphylococcal infection in one group can be excluded. The animals of each group were distributed into several different herds. Moreover, a large number of control animals in each herd did not show any rise of the serum titre.

Besides, the injection of vaccine was performed in such a manner and with such care that leakage after treatment must be excluded.

Consequently, a definite relationship existed between way of application and rise of the antistaphylolysin titre in blood serum. The result inspires to continue studying immunity response for longer periods of time after subcutaneous and intramuscular treatment, respectively.

Even if the antistaphylolysin titre hardly can be interpreted as an actual indicator of an organism's state of immunity, the aim of treatment with toxoids is generally, however, to obtain the greatest possible rise of the serum titre. On the basis of the present results, intramuscular application may be recommended as preferable to subcutaneous in toxoid treatment of cattle.

## SUMMARY

Two groups of cows were treated with a combined staphylococcal vaccine. In one group the vaccine was injected subcutaneously and in







the medium a yellowish hue which interfered with the reaction. The sterility was thereafter checked by incubation of the medium at 35-37° C for 24 hours.

In view of the observation by *E. Taylor* (1958) that dextrose phosphate substrate can in itself produce positive VP reactions by Barritt's method, the present author tested 104 batches of sterile medium in this respect. All batches produced a negative reaction by Barritt's method.

The following five kinds of peptone were used for testing the dextrose phosphate medium: 1. Wilson & Co., Chicago; 2. Witte, Rostock; 3. Orthana, Copenhagen; 4. Difco Proteose B 122, Detroit; 5. "indolefree" peptone, Schmitt-Jourdan, Boulogne-sur-Seine.

*Lactose peptone broth with indicator* (Liebig's extract 3 g, lactose 10 g, peptone Orthana 10 g, NaCl 3 g, 1 per cent bromocresol purple in alcohol 1 ml, and aq. dest. 1000 ml) was used for determining the production of acid and gas by coliform bacteria.

Two different methods were employed for inoculation of the cultures. In one the bacteria were grown first in broth, after which tubes containing dextrose phosphate were inoculated with 0.2 ml of this broth culture diluted to 10:1 (method A). In the other the material was transferred with a loop from agar slants or Endo agar plates to the dextrose phosphate tubes. The loop was dipped in the medium once (method B).

5 per cent  $\alpha$ -naphthol solution was used in 95 per cent ethyl alcohol since it produced stronger reactions than any kind of absolute alcohol solution. Numerous experiments showed that the first solution does not lose its strength after storage for one month at room temperature. The flasks were stoppered with screw on bakelite caps. The  $\alpha$ -naphthol (melting point 95-97° C) derived from Hopkin & Williams Ltd, England.

The material studied consisted of 1532 coliform strains from well water and 153 from faeces. Gram-negative non-sporing rods which fermented lactose under development of acid and gas were regarded as coliform strains.

## EXPERIMENTAL

Experimental studies have shown that the kind of peptone used in the dextrose phosphate medium, the temperature and time of incubation, and the testing technique, all play an important part. The experiments were often made with several simultaneous goals in view. To simplify the presentation of the results, each question will be dealt with separately below.

*Different kinds of peptone* The original recommendation, by *Clark & Lubs* (1915), was that Witte peptone alone should be used for the MR test. The same peptone was used for the VP test. Later other kinds of peptone were suggested. For the choice of the best kind of peptone, five peptones in dextrose phosphate medium were compared in the cultivation of 258 coliform strains inoculated by method A. The strains were grown at nine incubation temperatures between 27° and 45° C. The results are shown in Table 1. Differences between the various peptones, though slight, were observed even at 27° C. Peptone 1 gave the largest number of positive VP reactions in strains both from well water and from faeces. Peptone 5 gave almost as many positive reactions, peptones 3 and 4 a smaller, and peptone 2 the smallest number. The differences between the peptones were most manifest at higher incubation temperatures. At 45° C incubation of the faeces strains, acetoin was best formed in dextrose phosphate with peptones 1 and 5, but not at all with peptone 2. The best types of peptone were considered to be 1 and 5.

TABLE 1

Different Kinds of Peptone Number of Strains Showing Positive VP Reactions

	Kinds of peptone	Incubation temperatures								
		27°	30°	33°	35°	37°	39°	41°	43°	45°
218 strains from water	1	198	196	194	189	177	7			
	2	192	184	180	162	123	9			
	3	196	192	188	169	146	22			
	4	190	189	186	168	138	24			
	5	196	196	193	183	172	22			
40 strains from faeces	1	39	39	38	36	35	34	20	19	16
	2	37	36	33	24	20	10	1		
	3	37	36	37	36	35	20	12	10	6
	4	38	36	36	34	29	21	13	14	8
	5	38	37	37	36	34	28	17	17	14

Negative reactions with faint copper colouration occurred in certain strains cultivated in dextrose phosphate, usually with peptone 1. The higher the incubation temperature, the more vivid was the copper colour of the strains.

*Incubation temperature* As seen from Table 1, incubation at 27° C gave a larger number of positive VP reactions than at 30° C and above, irrespective of the type of peptone used in the dextrose phosphate medium.

Tests by method B were also made on 126 coliform strains isolated from water. The results confirmed that culture at 27° C gives the largest number of positive VP reactions.

Positive reactions seldom ceased suddenly at the transition from lower to higher incubation temperatures. As a rule very weak light pink reactions were observed between positive and negative reactions (e.g. 33° C + + +, 35° C ±, 37° C —). The VP reaction was usually found to be negative when no macroscopically visible growth occurred in the culture, but also at a high incubation temperature, despite the sometimes strong growth in the culture.

Coliform bacteria from faeces produced varying patterns of positive VP reactions. Some strains showed positive reactions up to 45° C, others formed acetoin at 43°, 41° or lower temperatures. Certain faeces strains produced acetoin only at 27° C. Coliform bacteria from water showed positive reactions only up to 39° C or below.

*Incubation period* To ascertain the most appropriate period of incubation for the VP test, 108 coliform strains from water were examined by method A. Each strain was inoculated into four tubes containing dextrose phosphate which were then incubated at 27° C. After 24, 48, 72 and 96 hours of incubation, respectively, one tube was removed from the incubator and examined for VP reaction. The results are shown in Table 2.

TABLE 2  
Incubation Period for VP Test

Days of incubation	1	2	3	4	Number of strains
+	+	+	+	+	141
—	—	—	—	—	1
—	—	—	+	+	5
+	+	+	—	—	2
—	—	—	—	—	36
Total positive reactions	143	153	157	155	
Total negative reactions	50	41	36	38	
	193	193	193	193	193

Incubation for so long a time as four days is inadvisable owing to the risk of splitting of acetoin (*Paine* 1927, *Williams et al* 1928, and *Tittler* 1938). The choice remains between two and three days of incubation. To settle this point, 1066 coliform strains from well water were inoculated by method B after membrane filtering and isolation on Endo agar plates. The highest and lowest incubation temperatures for acid and gas formation, were determined and the IMViC test was performed for each strain. Two tubes were then inoculated and incubated at 27° C. One tube was examined for acetoin formation after two and the other after three days. All strains except seven showed similar results after two and three days of incubation. These seven strains (0.6 per cent) were retested by method A. four were found to correspond to the results of two days and three strains to three days of incubation at the first examination. From the practical point of view, therefore, there was no difference between two and three days of incubation.

The conclusion was that two or three days of incubation at 27° C is the optimum period for the VP test.

*Technique of VP test.* In the experiments described below, each strain was inoculated by method A into five dextrose phosphate media containing different kinds of peptone and were incubated at 27°, 30°, 35° and 37° C for two days. Similar series consisting of one millilitre of each culture were transferred to two or more tubes for VP testing.

A comparison was first made of two modifications of Barritt's method. Barritt found that an addition of 0.6 ml of a 5 per cent solution of  $\alpha$ -naphthol and 0.2 ml of a 40 per cent solution of KOH to 1 ml of culture is as effective as the addition of 0.5 ml of a 11 per cent solution of  $\alpha$ -naphthol and 0.5 ml of a 16 per cent solution of KOH. Parallel investigations showed that the first modification gives rather more positive reactions than the second. The difference is most marked with peptones 2, 3 and 4 at 37° C incubation, the temperature chosen by Barritt for his investigations. Barritt's first modification was therefore chosen for the subsequent investigations.

In performing the VP test one must pay consideration to the factors which favour the reaction namely agitation and heating. Stirring also accelerated the reaction but was not usable in practice. On repeated agitation the colour of the reaction becomes darker after each shaking especially in the upper part of the mixture. Shaking of the tubes every tenth minute for the first 30 minutes proved to be sufficient. The largest number of positive reactions was obtained when the mixtures were left standing for another 30 minutes then reshaken and read.

To shorten the time before reading of the VP reaction experiments were made with tubes of larger gas absorbing area 20 mm instead of 15 mm diameter. Both tubes were shaken every tenth minute for 30 minutes. Tubes of 20 mm diameter were read after 30 minutes and 15 mm tubes after 1 hour. Table 3 shows that the percentage of positive reactions was greater in 20 mm than in 15 mm tubes. All types of peptone at four incubation temperatures gave a larger number of positive reactions in 20 mm tubes. This may be explained by the fact that the ingredients were mixed with air more easily and better in 20 mm than in 15 mm tubes.

Using the agitation method with tubes of 20 mm diameter the reaction mixture must be shaken every tenth minute i.e. four times in thirty minutes. Heating was employed to reduce the work involved. Serial experiments were made with cultures from 63 coliform strains dispensed in 1 ml portions in two tubes of 20 mm diameter. One series was shaken four times in the course of 30 minutes at room temperature and read. The other was shaken once placed in incubators at 45° C reshaken after 30 minutes and read. The results showed close agreement in cultures grown at 27° C.

*Comparison with some officially recommended methods of VP testing*  
In view of these investigations it may be said in advance that the techniques prescribed in *The Intern Standards for Drink Water of WHO* (1958), *The Standard Methods of USA* (1960) and *The Bact Ex of Wat Sup* (1956) will not yield satisfactory results. The three methods were nevertheless compared with the agitation method. All tests were made in tubes of 15 mm diameter. The agitation method was found to be superior to the other three methods in respect of the number of positive reactions for all kinds of peptone and all incubation temperatures.

*Inoculation technique* By parallel inoculations with different numbers of bacteria on dextrose phosphate media and titration of 48 hour cultures for acetoin content it was found that the strongest VP reactions occurred after inoculation with a large number of bacteria e.g. 20 million or a full loop of slant agar culture. The addition of a low number of bacteria inoculation by once dipping the loop with little material gave positive though weaker reactions.

*Control of VP reactions by means of acetoin* Acetoin (Fairmount Chemical Corp. New York 6) and acetyl methyl carbinol (L. Leight &

TABLE 2  
Incubation Period for VP Test

Days of incubation	1	2	3	4	Number of strains
	+	+	+	+	141
	—	+	+	+	11
	—	—	+	+	5
	+	+	+	—	2
	—	—	—	—	36
Total positive reactions	143	153	157	155	
Total negative reactions	50	41	36	38	
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Incubation for so long a time as four days is inadvisable owing to the risk of splitting of acetoin (Paine 1927, Williams *et al* 1928, and Tittler 1938). The choice remains between two and three days of incubation. To settle this point, 1066 coliform strains from well water were inoculated by method II after membrane filtering and isolation on Endo agar plates. The highest and lowest incubation temperatures for acid and gas formation, were determined and the IMViC test was performed for each strain. Two tubes were then inoculated and incubated at 27° C. One tube was examined for acetoin formation after two and the other after three days. All strains except seven showed similar results after two and three days of incubation. These seven strains (0.6 per cent) were retested by method A. Four were found to correspond to the results of two days and three strains to three days of incubation at the first examination. From the practical point of view, therefore, there was no difference between two and three days of incubation.

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**Technique of VP test** In the experiments described below, each strain was inoculated by method A into five dextrose phosphate media containing different kinds of peptone and were incubated at 27°, 30°, 37° and 37° C for two days. Similar series consisting of one millilitre of each culture were transferred to two or more tubes for VP testing.

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Co Ltd Colnbrook England) were used for these controls. The preparations gave exactly the same results in all tests.

A comparison was first made between 1) Voges Proskauer's original method with addition of 5 ml of 40 per cent KOH to 5 ml of culture reading after 24 hours 2) O'Meara's method with addition of 20 mg of creatine and 5 ml of 40 per cent NaOH to 5 ml of culture reading after 1 hour and 3) Barritt's method reading 1 hour after the addition of the ingredients (agitation 5 times).

Falling concentrations of both preparations were tested (Table 4). Dilution of acetoin in aq. dest. 0.5 per cent dextrose or 0.5 per cent  $\text{K}_2\text{HPO}_4$  gave no reaction. Dilutions of 0.5 per cent peptone water (each type of peptone separately) produced reactions by all three methods respectively dextrose  $\text{K}_2\text{HPO}_4$  and dextrose phosphate media (each type of peptone separately) produced reactions by all three methods. The results of these investigations with dextrose phosphate medium are shown in Table 4. Barritt's method was superior to the others acetoin being detected in dilutions of 1/500 000 by Barritt's but only in dilutions of 1/50 000 by the original VP and O'Meara's methods. O'Meara (1931) himself stated that his method reveals acetoin in dilution of at most 1/50 000. The reactions were considerably stronger with Barritt's than with the other methods. At dilutions of 1/10 and 1/100 the medium developed a yellow colour. Dextrose phosphate medium with peptone 2 produced rather weaker reactions in repeated tests which shows that the type of peptone may affect the reaction even without the influence of the bacteria.

Comparative tests of the agitation method were made in tubes of 10 and 20 mm diameter as well as of the heating method with different concentrations of the two acetoin preparations. The same technique as already recommended was used for these tests. The three methods gave exactly the same results as shown in Table 4 for Barritt's method. Peptone 2 again showed rather weaker reactions which were not improved even after heating.

*Titration of dextrose phosphate cultures for acetoin content.* A study was made of the quantity of acetoin in cultures of 100 VP positive coliform strains grown at 27° C for two days. The VP tests were carried out by the heating modification on undiluted cultures and on dilutions of the cultures in aq. dest. in the proportions 1:2, 1:5, 1:10, 1:20, 1:30 and 1:40. Two strains gave positive reactions only in undiluted cultures, four up to dilution 1:2, 29 up to 1:5, 50 up to 1:10, 14 up to 1:20 and one strain up to 1:30. The next higher dilutions usually showed ± reactions. All strains gave negative reactions in dilution 1:40. The differences in acetoin content had no relation to the ability of the strains to produce gas at 45° C or lower incubation temperature. Nor was there any correlation to type of bacteria (Aerogenes, intermediate or irregular) nor to origin of strains from water or faeces.

A comparison was made between Barritt's and O'Meara's methods

TABLE 3  
Procedure for VP Test Per Cent Positive VP Reactions in 5% Coliform Strains from Water

Incubation temperatures		27°					30°					37°				
Kinds of peptone		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Size of tubes		Read after min														
15 mm	60	91	82	85	72	91	91	70	83	76	91	89	59	69	63	28
20 mm	30	94	85	87	76	93	93	76	85	78	93	89	57	72	70	87
												78	50	65	57	72

TABLE 4  
Comparison of Three Methods for Detection of Acetoin in Dextrose Phosphate Medium

Dilutions of acetoin	Rep tone	1 10	1 100	1 1000	1 10 000	1 25 000	1 50 000	1 75 000	1 100 000	1 250 000	1 500 000	1 750 000
Original VP	1-5	yellow	yellow	±	+	±	+	+	+	+	+	+
O'Leary	1 5	yellow	±	+	±	±	±	±	±	±	±	±
Barritt	1	+	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+	+

++ bright raspberry or ruby red  
 + weaker raspberry or ruby red  
 + aniline or light red  
 ± very faint pink  
 — colourless or copper coloured

Co Ltd, Colnbrook, England) were used for these controls. The preparations gave exactly the same results in all tests.

A comparison was first made between 1) Voges-Proskauer's original method with addition of 5 ml of 40 per cent KOH to 5 ml of culture, reading after 24 hours, 2) O'Meara's method with addition of 25 mg of creatine and 5 ml of 40 per cent NaOH to 5 ml of culture, reading after 1 hour and 3) Barritt's method, reading 1 hour after the addition of the ingredients (agitation 5 times).

Falling concentrations of both preparations were tested (Table 4). Dilution of acetoin in aq. dest., 0.5 per cent dextrose or 0.5 per cent  $K_2HPO_4$  gave no reaction. Dilutions of 0.5 per cent peptone water (each type of peptone separately) produced reactions by all three methods respectively, dextrose,  $K_2HPO_4$ , and dextrose phosphate media (each type of peptone separately) produced reactions by all three methods. The results of these investigations with dextrose phosphate medium are shown in Table 4. Barritt's method was superior to the others,

most 1/50,000. The reactions were considerably stronger with Barritt's than with the other methods. At dilutions of 1/10 and 1/100 the medium developed a yellow colour. Dextrose phosphate medium with peptone 2 produced rather weaker reactions in repeated tests, which shows that the type of peptone may affect the reaction even without the influence of the bacteria.

Comparative tests of the agitation method were made in tubes of 15 and 20 mm diameter, as well as of the heating method, with different concentrations of the two acetoin preparations. The same technique as already recommended was used for these tests. The three methods gave exactly the same results as shown in Table 4 for Barritt's method. Peptone 2 again showed rather weaker reactions which were not improved even after heating.

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A comparison was made between Barritt's and O'Meara's methods



TABLE 5  
*Typing of 21 Coliform Strains by Barritt's and O'Meara's Methods*

Number of strains	Barritt				O'Meara			
	I	M	V	C	I	M	V	C
4	+	+	+	+	+	+	—	+
4	—	+	+	+	—	+	—	+
5	—	—	+	+	—	—	—	+
4	+	—	+	+	+	—	—	+
1	—	+	+	—	—	+	—	—
2	+	+	+	—	+	+	—	—
1	—	—	+	—	—	—	—	—

in the same culture, 40 strains being tested. All strains which, by Barritt's method, gave positive reactions only in undiluted cultures or in dilutions up to 1/2 or 1/5, showed negative reactions by O'Meara's method. Only strains which produced a higher acetoin content from dilutions of 1/10 and upwards gave  $\pm$  and + reactions also by O'Meara's method. In this investigation as well, O'Meara's method proved some ten times less effective than Barritt's.

The effect on the typing result of the use of O'Meara's method compared with Barritt's is shown in Table 5. It is seen that three strains which by O'Meara's method would be typed as *Esch. coli* did not produce acid and gas at 45° and did not form typical *Esch. coli* colonies on Endo agar. By Barritt's method, on the other hand, the strains would be regarded as irregular.

## DISCUSSION

The purpose of this study has been to evolve a method of performing the VP test which is characterized by the greatest possible measure of standardization and sensitivity. The study confirmed the long known fact that Barritt's method is more sensitive than other generally used methods (Barritt 1936, Levine 1941, Batty-Smith 1941, Kauffmann 1954, Schubert 1956, Snassuna et al 1960, *The Bacteriological Examination of Water Supplies* 1956, WHO 1958, *The Standard Methods of USA* 1960, etc.). According to *The Bacteriological Examination of Water Supplies* and Taylor, E (1958), however, Barritt's method is considered too sensitive, and O'Meara's method, which is ten times less sensitive, is suggested instead. The reason given is that "the high sensitivity of the Barritt's test may tend to obscure differences between members of the coliform group" (*The Bact. Ex.*) and that *Esch. coli* "may give positive results with the more sensitive reagents" (Taylor, E 1958). The present author has had several reasons for wishing to increase the sensitivity of the reaction. In the first place a sensitive method is desirable for the detection of even small quantities of acetoin in biochemical bacterial examinations. In the second place a sensitive method is preferable also for the typing of coliform bacteria. The production of acetoin is subject to altogether too many varying factors to justify the

necessity for the production of a large quantity as criterion in routine typing

The type of peptone used in the dextrose phosphate medium has a considerably greater rôle in the VP than in the MR test. For the choice of the best type of peptone it proved advisable to take several VP-positive strains from faeces and grow them on dextrose phosphate media with different kinds of peptone at up to 45° C. It must be remembered, however, that all types of peptone are not universally available and that the quality of the selected peptone may vary. On practical grounds it is desirable to use the same medium both for the VP and for the MR tests. The type of peptone should therefore be chosen with greater care than has been done previously.

The incubation temperature is still an open question. Incubation at 27° C was found to give the greatest number of positive VP reactions also with coliform strains from faeces. The difference between the peptone varieties is least at 27° C.

The present investigations showed two or three days of incubation to be best for the VP test. It is thus possible to carry out VP and MR tests simultaneously on the third day of incubation, or VP on the second and MR on the fourth day if the third day happens to be a holiday.

### CONCLUSION

The VP and MR reactions can be tested on the same culture. The following medium, reagents and methods are suggested.

Medium	Dextrose	5 g
	Peptone	5 g
	K <sub>2</sub> HPO <sub>4</sub> (anhydrous)	5 g
	Aq. dest.	1000 ml

Peptone and K<sub>2</sub>HPO<sub>4</sub> are dissolved in aq. dest. for 20 minutes in steam. Dextrose and aq. dest. are then added to make up 1000 ml. The medium is filtered, distributed in 4–5 ml portions into tubes and sterilized in steam, but not for longer than 20 minutes. The tubes should not come into contact with the hot condensed water which may collect during the autoclaving. The sterility is checked by incubation for 24 hours at 35°–37° C.

- Reagents
- 1 0.1 g of methyl red is dissolved in 250 ml 95 per cent ethyl alcohol. Alternatively, according to *Clark & Lubs*, 0.1 g of methyl red is dissolved in 300 ml of 95 per cent ethyl alcohol to which 200 ml of aq. dest. is added.
  - 2 50 g of  $\alpha$ -naphthol is dissolved in 100 ml of 95 per cent ethyl alcohol and kept in a dark flask with ground glass stopper or screw-on cap for not more than 1 month.
  - 3 40 per cent KOH solution.

## Methods

**VP test** Dextrose phosphate medium is inoculated with a loopful of material and incubated at 27° C. After two or three days 1 ml of culture is transferred to a tube of about 20 mm diameter, after which 0.6 ml of  $\alpha$ -naphthol solution and 0.2 ml of KOH solution are added. The tube is shaken vigorously three times in 30 minutes and the reaction is noted. The same result is obtained if the tubes are shaken, incubated at 45° C, reshaken after 30 minutes and read.

**MR test** The rest of the culture is replaced in the incubator at 27° C if the VP test was made after 48 hours of incubation. On the next day or two days later, four drops of methyl red solution are added to the culture and the reaction is read within 15 minutes.

## SUMMARY

A study has been made of the significance of various technical factors on the sensitivity of the VP test. In the titration of dextrose phosphate cultures, Barritt's method proved ten times more sensitive than O'Meara's. Barritt's method should therefore be employed for the VP test as being the most sensitive.

The type of peptone used in the dextrose phosphate medium is of greater significance in the VP than in the MR test. Care should therefore be taken in the choice of peptone. It is desirable that the medium should be suited for both the VP and the MR test.

The best incubation temperature for the VP test is 27° C, i.e. the same temperature as for the MR test. The period of incubation for the VP test should be two or three days.

The VP reaction can be read 30 minutes after addition of the reagents provided that the mixture is repeatedly shaken in tubes of about 20 mm diameter or is heated to 45° C.

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## A SIMPLE METHOD TO PREPARE PARTICLES IN SUSPENSION FOR ELECTRON MICROSCOPY

By

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Received 7.1.62

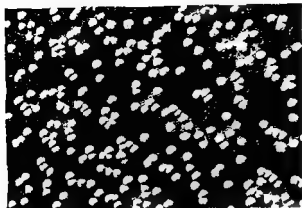
A great number of different methods have been developed to prepare particles from suspensions for electron microscopy. They range from more exact and troublesome methods, where particular care is taken for the preservation of the three-dimensional structure (Anderson 1950, Williams 1953), to less subtle but simpler ones, where the suspensions are subjected to air-drying (e.g. Valentine & Bradfield 1954, Hobbs & Hodgkiss 1960). We want to describe a simple method not found in recent handbooks (Anderson 1956, Reimer 1959, Kay 1961) which we have now routinely used for over three years.

A formvar-coated copper grid (Athene) is taken with a forceps, turned upside down, and the formvar-surface touched to the surface of the suspension to be investigated (usually a faintly turbid suspension, approximately  $10^8$ – $10^9$  particles/ml, kept in a test tube). When the forceps is raised, a drop of the liquid sticks to the grid. The size of this drop is reduced by touching the surface of the drop to the test tube wall or to the surface of the suspension. This is most easily achieved when the angle between the grid and the surface to be touched is nearly  $90^\circ$ . If the particles to be investigated are suspended in a liquid with high surface tension, e.g. distilled water or salt solutions, the whole drop is often removed and few particles are left on the grid. Suspensions with low surface tension leave an evenly moistened surface which rapidly dries, and the particles are evenly distributed over the grid. Particle suspensions from cells often have low surface tension due to the effect of macromolecules of cellular origin. The same effect is achieved by addition of gelatine (final conc. 0.01 per cent) (Williams & Fraser 1953). A low surface tension of the suspension medium also reduces the flattening of the particles by surface tension forces during drying (Anderson 1952). If salts must be present, volatile ones (e.g. ammonium acetate) (Backus & Williams 1950) are preferred.

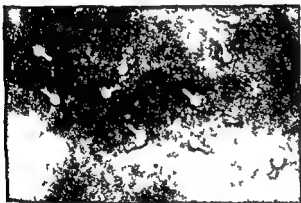
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*Fig 1* Polystyrene  
Latex spheres (Dow  
Chemical Co )  
( $\times 20\,000$ ) Chromium  
shadowed



*Fig 2* T2 phages  
( $\times 24\,000$ ) Shadowed  
with platinum  
palladium



*Fig 3* Cell walls from  
*Streptococcus*  
pyogenes type 12  
( $\times 8\,000$ ) (chromium  
shadowed)

Ordinary copper grids have routinely been used "Positively charged grids (Ribi *et al* 1960) also give good results but have not been found necessary for our method. When examination of the three dimensional structure of the specimen is not so important, the method described above has been preferred, because it is rapid, simple and easily reproduced.

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## SUPPLEMENT TO THE KAUFFMANN-WHITE-SCHEME (VI)

By

F KAUFFMANN

Received 4:63

This paper is the 2 supplement to the *Kauffmann-White-Schema* given in the book "*Die Bakteriologie der Salmonella-Species*" (F Kauffmann 1) and the 6 supplement to the review "*Das Kauffmann-White-Schema*" (F Kauffmann 2). It contains 79 new *Salmonella* species and 8 variants of known species recognized during 1962. From the 79 new species 47 belong to sub-genus I and 32 to sub-genus II. The species of sub-genus II are indicated by an asterisk (\*).

Deviating biochemical results, not given in the table were obtained in the following species:

\**S. sofia* fermented salicin after 1 day

\**S. kulsriwier* fermented salicin after 1 day and produced indole

*S. brybhumii* did not ferment maltose and *S. uhlenhorst* fermented it delayed and irregularly

\**S. lincoln* fermented salicin after 4 days

\**S. sachsenwald* was KCN positive

A culture of *S. karamoja* containing the O 1 antigen was found = 140 z<sub>11</sub> 1,2

One culture of *S. pleuten* did not ferment salicin and was indole-negative. The original culture fermented salicin after 1-2 days and was indole-positive

\**S. urindaban* did not ferment sorbitol

\**S. parera* fermented salicin and maltose after 3 days and was KCN positive. This species belongs to a special group of species, called "atypical sub-genus II" by F Kauffmann (4) in a paper "*Zur Differentialdiagnose der Salmonella Sub-Genera I, II und III*" which is in press.

To this special, biochemical group belong further the following 6 species of Table 1 in the paper "*Supplement to the Kauffmann-White-Schema (V)*": *S. soesterberg*, *S. ...*, *S. ...*, *S. ...*, *S. ...*, *S. ...*. These species are ... reactions in dulcitol, ... reactions in KCN, gelatin and salicin, but *S. tuindorp* was salicin negative



*S soesterberg* = 21 z<sub>1</sub> z<sub>3</sub> - = Ar 2959 55 = Ar 22 1 2 5 6  
*S houten* = 43 z<sub>1</sub> z<sub>3</sub> - = Ar 1450 53 = Ar 21 1 2 6  
*S wassenaar* = 50  $\Sigma$  (p) z<sub>51</sub> - = Ar \ 99 = Ar 9a 9b 13 1b

Further the following Arizona species belongs to these atypical *Salmonella* sub genus II cultures

Ar 396-56 = 10a 10c 13 15 = Sal 40 G -

*S abony* var *haifa* = 4 12 b e n \ differs from the original *S abony* culture = 4 5 12 b e n \ also biochemically since l tartrate is fermented by var *haifa* promptly by the original culture delayed and irregularly

*S sofia* var = 4 12 27 b - is closely related to *S sofia* = 4 12 b It is not investigated as yet whether the H antigens are identical or not While *S sofia* fermented d tartrate after 2 days this substance was not fermented by 4 12 27 b -

Further in contrast to *S sofia* 4 12 27 b - did not ferment salicin promptly

*S kingston* var *copenhagen* = 4 12 g s t - differs from the original *S kingston* culture = 1 4 12 27 g s t - also biochemically since inositol and l tartrate are fermented promptly by var *copenhagen* by the original culture inositol is not fermented and l tartrate delayed and irregularly

*S travis* = 4 5 12 g (p) z<sub>1</sub> 17 P R Edwards McWhorter & Douglas who described this species identified the special H antigen z<sub>51</sub> which also is present in *S alamo* *S maritima* *S new mexico* and *S wayne* According to R Rohde (personal communication) it is necessary to absorb the g (p) z<sub>1</sub> serum by *S dublin* + *S moscow* + *S budapest* + *S berla* The author has confirmed these results

*S lindrick* var 17 = 9 12 e n \ 17 This culture which was received from Dr J Taylor London differs from the original *S lindrick* culture - 9 12 e n \ 15 7 also biochemically since inositol was fermented by the new strain

*S rutgers* 3 10 1 z<sub>10</sub> 17 was omitted from the K W scheme since 1 z<sub>10</sub> is regarded as an R phase Consequently 2 new types received from Dr R Rohde Hamburg *Salmonella* 3 10 1 z<sub>1</sub> 15 and 9 12 1 z<sub>10</sub> 15 were not added to the K W scheme (in agreement with Dr Rohde)

A new *Salmonella* serotype *Salmonella cook* (39 z<sub>1</sub> 15) containing an undescribed flagellar antigen was published by McWhorter Douglas & Edwards but not added to the K W scheme by the author since the H antigen z<sub>5</sub> could be in R phase of *S champagne*

Further the following diphasic cultures were isolated

*S bleedon* = 17 f l t e n \ z<sub>1</sub>

*S wangata* ~ 19 12 z<sub>1</sub> z<sub>3</sub> 17

*S lunni* = 43 z<sub>1</sub> 15 7





TABLE (cont.)

Species	Ara	Dul	Ino	Rha	Tre	Xyl	Cly	H <sub>2</sub> S	cel	d	l	l	Ch	Muc	Mol
<i>S. arbutus</i> — 43 p 14	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. cluven</i> — 44 f g	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. uhlenhorst</i> — 44 a 1 w	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. trin lobat</i> — 45 a c h x	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. rje la</i> — 45 a g 10	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. strellor</i> — 45 f g	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. 45 g m</i>	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. bremer</i> — 45 g m s t e n x	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. perinet</i> — 45 m t c n x z 10	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. kloz muls</i> — 45 x z 10	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. stelling</i> — 47 d e n x	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. montine</i> — 47 j 16	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. cherana</i> — 47 x 20	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. hammonia</i> — 48 c n x z 15 21	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. hoogruen</i> — 50 z 10 z 6 z 42	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. meakin</i> — 51 c h 12	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. 51 - 17</i>	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. manamba</i> — 57 p 10 c n x z 15	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. betinku</i> — 59 k (x)	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—
<i>S. lutan</i> — 60 x c n x	+	+	—	+	+	+	+	+	—	+	×	×	+	+	—

K<sub>2</sub> Ara — arabinose Dul — dulcitol Ino — inositol Rha — rhamnose, Tre — trehalose Xyl — xylose Gly — glycine  
 glycerol fuchsin broth Gel — gelatin d — d tartrate l — l tartrate Cit — sodium citrate, Muc — mucic  
 inacid Mal — sodium malonate Arabinose to xylose + — positive after 1 day — — negative after 30 days X — late  
 and irregularly positive or negative Stems glycerol fuchsin broth + + lilac after 12 days, — negative after 8 days  
 trehalin + slowly positive in ferr us chl ride serum and rapidly positive in the Kohn test modified by Lautrop Organic  
 acids + — positive after 1 day + 21 — positive after 21 days — — negative after 14 days — — sub gina 11

With regard to the new *Salmonella* O groups the following should be mentioned

- Salmonella* O 50 identical with Arizona 9a,9c
- Salmonella* O 51 identical with Arizona 1,2
- Salmonella* O 52 identical with Arizona 31
- Salmonella* O 53 related to Arizona 1,4
- Salmonella* O 56 identical with Arizona 14
- Salmonella* O 57 identical with Arizona 34
- Salmonella* O 58 identical with Arizona 1,3,3
- Salmonella* O 59 related to Arizona 19
- Salmonella* O 60 identical with Arizona 24

### *List of Salmonella Species Recognized in 1962*

- Salmonella abony* var *haifa* Kauffmann = 4,12 b c,n,x
- \**Salmonella acres* = 1,13,23 b z<sub>1</sub>
- Salmonella anderlecht* van Oye, Fievez, Granville & van Goethem = 3,10 c l,w Annal Inst Pasteur 107 931-932, 1962
- Salmonella arkansas* = (3),(15),34 c,h 1,5
- Salmonella assen* = 21 a -
- Salmonella barmbek* Rohde & Bischoff = 16 d z<sub>0</sub>
- Z Hyg in press
- \**Salmonella beloha* nom nov Kauffmann  
(*Salmonella* beloha Le Minor, Le Noc & Coynault) = 18 z<sub>1</sub> -  
Bull Soc Path exot 55 216-220, 1962
- \**Salmonella betioky* nom nov Kauffmann  
(*Salmonella* betioky Le Minor, Le Noc & Coynault) = 59 k (z)  
Bull Soc Path exot 55 216-220, 1962
- \**Salmonella bleadon*, diphasic = 17 f,g,l c,n,x,z<sub>1</sub>
- \**Salmonella bremen* = 45 g m,s,t c,n,x (45<sub>1</sub>,45<sub>2</sub>)
- Salmonella briybhumi* = 11 i 15
- Salmonella broughton* = 1,3,19 b l,w
- \**Salmonella bunnik*, diphasic = 43 z<sub>1</sub> 1,5,7 (43<sub>1</sub>,43<sub>3</sub>,43<sub>1</sub>)
- Salmonella burgas* = 16 l,x c,n,z<sub>1</sub>
- \**Salmonella caledon* Brede = 4,12 g,m c,n,x  
Annal Inst Pasteur 107 933-934, 1962
- \**Salmonella calvinia* = 6,7 a z<sub>1</sub>
- \**Salmonella carletonville* = 38 d
- Salmonella chagana* = 1,13,23 n 1,5
- \**Salmonella chersina* = 47 z z<sub>1</sub> (47<sub>1</sub>,47<sub>3</sub>)
- Salmonella congo* = 13,23 g,l
- \**Salmonella durbanville* = 4,12 z<sub>3</sub> 1,5,7
- \**Salmonella ejeda* nom nov Kauffmann  
(*Salmonella* ejeda Le Minor, Le Noc & Coynault) = 45 n z<sub>1</sub>  
(45<sub>1</sub>,45<sub>3</sub>) Bull Soc Path exot 55 216 220 1962

*Salmonella finkenwerder Rohde & Tiedje* = 161425 d 10

Z Hyg in press

*Salmonella fisch erhuette* — 16 a enz15

*Salmonella gojenberg Rohde & Muller* = 11324 gt 15

Z Hyg in press

*Salmonella goodu ood* — 1322 z3 enz

*Salmonella grabouw* — 11 g m s t z30

*Salm nella grunty* = 140 z30 16 (140:403)

*Salmonella hammonia Rohde & Muller* = 48 enz1 z (48:48)

Z Hyg in press

*Salmonella harvestehude Rohde & Tiedje* = 142 y z0

Z Hyg in press

*Salmonella heeren* — 11 i 16

*Salmonella heron* — 16 a z

*Salmonella hooggraven* — 50 z0 z3 z1 (51:503)

*Salmonella inprau* = 41 z0 enz

*Salmonella igrigny* = 43 z3 (43:43)

*Salmonella isuge* = 1323 d z3

*Salmonella kaitaan* = 161420 m t

*Salmonella karamoja* with O 1 antigen ~ 140 z41 19 (140:40)

*Salmonella kingsdon var copenhagen kauffmann* — 419 g s t

*Salmonella klapmuts* = 45 z z30 (45:432)

*Salmonella kralingen* — (8) 20 y z0

*Salmonella kuisgraver* — 1912 g m s t enz

*Salmonella ligna* — 37 z0 z6

*Salmonella lincoln* = 11 m t enz

*Salm nella lin trich var 1* — 912 enz 17

*Salmonella lokstedt Rohde & Tiedje* — 1319 1 z3 z4 12

Z Hyg in press

*Salmonella lul n* — 60 z enz

*Salmonella maastricht* — 11 z41 12

*Salm nella ma sujuri Rohde & Tiedje* — 1319 fgt enz3

Z Hyg in press

*Salmonella manombo nom nov kauffmann*

(*Salmonella manomila* Le Minor Le Soc & Coignault) —

z30 enz z3 Bull Soc Path exot 70 216-220 1969

*Salmonella maron* 310 d z3

*Salm nella massak ry* = 30 r 1 w

*Salmonella menha len* = (3) (15) 71 1 z 17

*Salm nella meshin* = 51 e h 1 z

*Salm nella montian Ope & Vechelpul* = 1412 d 1 w

Ann d Inst Pasteur in press

*Salm nella m natine* = 47 v 16 (47:473)

*Salm nella ngor nom nov kauffmann*

(*Salmonella ngor* Le Minor Chamlon Bories Mart & Charie-Mar

vances) — 1319 1 v 1 Bull Soc Path exot 70 713-715 1962

With regard to the new *Salmonella* O groups the following should be mentioned

- Salmonella* O 50 identical with Arizona 9a,9c
- Salmonella* O 51 identical with Arizona 1,2
- Salmonella* O 52 identical with Arizona 31
- Salmonella* O 53 related to Arizona 1,4
- Salmonella* O 56 identical with Arizona 14
- Salmonella* O 57 identical with Arizona 34
- Salmonella* O 58 identical with Arizona 1,33
- Salmonella* O 59 related to Arizona 19
- Salmonella* O 60 identical with Arizona 24

### *List of Salmonella Species Recognized in 1962*

- Salmonella abony* var *haifa* Kauffmann = 4,12 b e,n,\
- \**Salmonella acres* = 1,13,23 b z<sub>12</sub>
- Salmonella anderlecht* van Oye, Fievez, Granville & van Goethem = 3,10 c l,w Annal Inst Pasteur 103 931-932, 1962
- Salmonella arkansas* = (3),(15),34 c,h 1,5
- Salmonella assen* = 21 a -
- Salmonella barmbek* Rohde & Bischoff = 16 d zc
- Z Hyg in press
- \**Salmonella beloha* nom nov Kauffmann  
(*Salmonella beloha* Le Minor, Le Noc & Coynault) = 18 z<sub>11</sub> -  
Bull Soc Path exot 55 216-220, 1962
- \**Salmonella betioky* nom nov Kauffmann  
(*Salmonella betioky* Le Minor, Le Noc & Coynault) = 59 k (z)  
Bull Soc Path exot 55 216-220, 1962
- \**Salmonella bleedon*, diphasic = 17 f,g,t e,n,x,z<sub>10</sub>
- \**Salmonella bremen* = 45 g,m,s,t e,n,x (45<sub>1</sub>,45<sub>2</sub>)
- Salmonella brybhumu* = 11 i 1,5
- Salmonella broughton* = 1,3,19 b l,w
- \**Salmonella bunnik*, diphasic = 43 z<sub>12</sub> 1,5,7 (43<sub>1</sub>,43<sub>3</sub>,43<sub>1</sub>)
- Salmonella burgas* = 16 l,\ e,n,z<sub>10</sub>
- \**Salmonella caledon* Brede = 4,12 g,m e,n,\
- Annal Inst Pasteur 103 933-934, 1962
- \**Salmonella calvinia* = 6,7 a z<sub>11</sub>
- \**Salmonella carletonville* = 38 d -
- Salmonella chagoua* = 1,13,23 i 1,5
- \**Salmonella chersina* = 47 z z<sub>c</sub> (47<sub>1</sub>,47<sub>2</sub>)
- Salmonella congo* = 13,23 g,t -
- \**Salmonella durbanville* = 4,12 z<sub>39</sub> 1,5,7
- \**Salmonella ejeda* nom nov Kauffmann  
(*Salmonella ejeda* Le Minor, Le Noc & Coynault) = 45 a z<sub>10</sub>  
(45<sub>1</sub>,45<sub>2</sub>) Bull Soc Path exot 55 216 220 1962

*Salmonella finckenwerder Rohde & Tiedje* — 16 14 25 d 15

Z Hyg in press

*Salmonella fischerhuetten* — 16 a enz<sub>15</sub>

*Salmonella gojenberg Rohde & Muller* — 1 13 23 gt 15

Z Hyg in press

*Salmonella goodwood* — 13 29 z<sub>9</sub> enz

*Salmonella grabouw* = 11 gm st z<sub>39</sub>

*Salmonella grunty* — 1 40 z<sub>39</sub> 16 (1 40<sub>1</sub> 40<sub>3</sub>)

*Salmonella hammonia Rohde & Muller* — 48 enz<sub>2</sub> z<sub>4</sub> (48<sub>1</sub> 48)

Z Hyg in press

*Salmonella harpestehude Rohde & Tiedje* — 1 42 y z<sub>6</sub>

Z Hyg in press

*Salmonella heerlen* — 11 i 16

*Salmonella heron* — 16 a z<sub>6</sub>

*Salmonella hooggraven* — 50 z<sub>0</sub> z<sub>6</sub> z<sub>4</sub> (51<sub>1</sub> 50<sub>3</sub>)

*Salmonella inprau* — 41 z<sub>0</sub> enz

*Salmonella irigny* — 43 z<sub>38</sub> (43<sub>1</sub> 43)

*Salmonella isuge* — 13 y<sub>3</sub> d z<sub>6</sub>

*Salmonella kaitaan* — 16 14 20 mt

*Salmonella karamoja with O 1 antigen* — 1 40 z<sub>11</sub> 12 (1 40<sub>1</sub> 40)

*Salmonella kingston var copenhagen kauffmann* — 4 12 gt

*Salmonella klappmuts* — 45 z z<sub>39</sub> (45<sub>1</sub> 45)

*Salmonella kralingen* — (8) 90 y z<sub>6</sub>

*Salmonella kuilsrivier* = 19 12 gm st enz

*Salmonella lugna* — 30 z<sub>0</sub> z<sub>6</sub>

*Salmonella lincoln* = 11 mt enz

*Salmonella lindrick var 1* 9 12 enz 17

*Salmonella lokstedt Rohde & Tiedje* = 13 19 1 z<sub>13</sub> z<sub>4</sub> 19

Z Hyg in press

*Salmonella luton* 60 z enz

*Salmonella maastricht* — 11 z<sub>11</sub> 19

*Salmonella mai luguri Rohde & Tiedje* = 13 19 fgt enz

Z Hyg in press

*Salmonella manombo nom nov kauffmann*

(*Salmonella manombl* o Le Minor Le Noc & Coynault) —

7 z<sub>4</sub> enz z<sub>3</sub> Bull Soc Path exot 50 216-290 1969

*Salmonella maron* — 3 10 d z<sub>3</sub>

*Salmonella massakory* = 30 r 1 w

*Salmonella mentaden* = (3) (15) 34 1 v 17

*Salmonella meskin* = 31 eh 19

*Salmonella m n a i a n Oye & Vechelput* = 1 4 12 d 1 w

Annal Inst Pasteur in press

*Salmonella m n a l i n e* = 47 v 16 (47<sub>1</sub> 47<sub>3</sub>)

*Salmonella n j o r nom noi kauffmann*

(*Salmonella ngor* Le Minor Chumpon Bories Marx & Charité Mar  
times) = 13 19 1 v 1 w Bull Soc Path exot 50 213 215 1962



With regard to the new *Salmonella* O groups the following should be mentioned:

- Salmonella* O 50 identical with Arizona 9a,9c
- Salmonella* O 51 identical with Arizona 1,2
- Salmonella* O 52 identical with Arizona 31
- Salmonella* O 53 related to Arizona 1,4
- Salmonella* O 56 identical with Arizona 14
- Salmonella* O 57 identical with Arizona 34
- Salmonella* O 58 identical with Arizona 1,33
- Salmonella* O 59 related to Arizona 19
- Salmonella* O 60 identical with Arizona 24

### *List of Salmonella Species Recognized in 1962*

- Salmonella abony* var *haifa* Kauffmann = 4,12 b c,n,\
- \**Salmonella acres* = 1,13,23 b. z<sub>12</sub>
- Salmonella anderlecht* van Oye, Fievez, Granville & van Goethem = 3,10 c l,w Annal Inst Pasteur 103 931 932, 1962
- Salmonella arkansas* = (3),(15),34 c,h 1,5
- Salmonella assen* = 21 a -
- Salmonella barmbek* Rohde & Birchhoff = 16 d z<sub>0</sub>
- Z Hyg in press
- \**Salmonella beloha* nom nov Kauffmann  
(*Salmonella beloha* Le Minor, Le Noc & Coynault) = 18 z<sub>10</sub> -  
Bull Soc Path exot 55 216 220, 1962
- \**Salmonella betioky* nom nov Kauffmann  
(*Salmonella betioky* Le Minor, Le Noc & Coynault) = 59 k (z)  
Bull Soc Path exot 55 216-220, 1962
- \**Salmonella bleedon*, diphasic = 17 f,g,t c,n,\,z<sub>13</sub>
- \**Salmonella bremen* = 45 g,m,s,t c,n,x (45<sub>1</sub>,45<sub>2</sub>)
- Salmonella brijbhumi* = 11 i 1,5
- Salmonella broughton* = 1,3,19 h l w
- \**Salmonella bunnik*, diphasic = 43 z<sub>12</sub> 1,5,7 (43<sub>1</sub>,43<sub>3</sub>,43<sub>1</sub>)
- Salmonella burgas* = 16 l,\ c,n,z<sub>13</sub>
- \**Salmonella caledon* Brede = 4,12 g,m c,n,x  
Annal Inst Pasteur 103 933-934, 1962
- \**Salmonella calvinia* = 6,7 \ z<sub>12</sub>
- \**Salmonella carletonville* = 38 d -
- Salmonella chagoua* = 1,13 23 a 1,5
- \**Salmonella chersina* = 47 z z<sub>c</sub> (47<sub>1</sub>,47<sub>3</sub>)
- Salmonella congo* = 13,23 g,t -
- \**Salmonella durbanville* = 4,12 z<sub>39</sub> 1,5,7
- \**Salmonella ejeda* nom nov Kauffmann  
(*Salmonella ejeda* Le Minor, Le Noc & Coynault) = 45 ■ z<sub>10</sub>  
(45<sub>1</sub>,45<sub>3</sub>) Bull Soc Path exot 55 216 220, 1962

*Addendum to Supplement 1*  
(Kauffmann 3)

*Salmonella gateshead* — (9) 46 g s t

*Salmonella jukestown* = 13 23 i e n z<sub>1</sub>

*Salmonella lobatsi* = 52 157

*Salmonella tejas* nom nov Kauffmann

(*Salmonella tejas* Heather Howard Dag & Bredthauer) — 4 12 z<sub>36</sub> —  
J Bacter 84 377 1962

*Salmonella travis* nom nov Kauffmann

(*Salmonella travis* Edwards McWhorter & Douglas) —

45 12 g (p) z<sub>1</sub> 17 Internat Bull Bact Nomencl & Taxon  
12 67 1962

*Salmonella wingrove* = 68 c 12

*Salmonella bonaire* was written by a mistake without the e

*Salmonella zeist* = 18 z<sub>10</sub> z<sub>6</sub> belongs to sub genus II

SUMMARY

A supplement to the Kauffmann White Scheme is given containing 79 new *Salmonella* species and 8 variants recognized during the year 1962

REFERENCES

- Edwards P R McWhorter A C & Douglas G W A new *Salmonella* serotype  
*Salmonella travis* (45 12 g (p) z<sub>1</sub> 17) and a redefinition of the H antigens of  
several *Salmonella* serotypes Internat Bull Bacter Nomenclat & Taxon  
12 67 0 1962
- Kauffmann F (1) Die Bakteriologie der *Salmonella* Species (Munksgaard Copen-  
hagen 1961)
- Kauffmann F (2) Das Kauffmann White Schema = Ergebnisse der Mikrobio-

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Internat Bull Bacter Nomenclat & Taxon 12 181 183 1962 " " " " "

- \* *Salmonella parera* = 11 z<sub>1</sub>, z<sub>23</sub> -
- \* *Salmonella perinet* nom nov Kauffmann  
(*Salmonella perimet* Le Minor, Le Noc & Coynault) =  
45 m, t c, n, x, z<sub>15</sub> (45<sub>1</sub>, 45<sub>7</sub>, 45<sub>3</sub>) Bull Soc Path exot 55 216 220  
1962
- Salmonella putten* = 13, 23 d l, w
- Salmonella riggii* = 6, 7 g, t -
- Salmonella rissen* Rohde & Tiedje = 6, 7 f, g -  
Z Hyg in press
- \* *Salmonella sachsenwald* Rohde & Muller = 1, 40 z<sub>1</sub>, z<sub>23</sub> - (40<sub>1</sub>, 40<sub>40</sub>)  
Z Hyg in press
- Salmonella santhiaba* nom nov Kauffmann  
(*Salmonella santhiaba* Le Minor, Chambon, Bories, Marx & Charie  
Marsaïnes) = 40 l, z<sub>23</sub> 1, 6 (40<sub>1</sub>, 40<sub>1</sub>) Bull Soc Path exot 55 213-  
215, 1962
- Salmonella schalkwijk* = (6), 14, (24) i c, n
- Salmonella sekondi* = 3, 10 c, h z<sub>6</sub>
- \* *Salmonella sofia* Wesselinoff & Dimow = 4, 12 h  
Zbl Bakter I Orig 187 263 265, 1962
- \* *Salmonella sofia* var 27 = 4, 12, 27 h -  
*Salmonella stellingen* Rohde & Bischoff = 47 d c, n, x (47<sub>1</sub>, 47<sub>3</sub>)  
Z Hyg in press
- \* *Salmonella stevenage* = 1, 13, 23 z<sub>1</sub> 1, 7
- Salmonella suelldorf* Rohde & Weissflog = 45 f, g (45<sub>1</sub>, 45<sub>1</sub>)  
Z Hyg in press
- Salmonella tarshyne* = 9, 12 d 1, 6
- \* *Salmonella tulcar* = 6, 8 a z<sub>2</sub>
- Salmonella uhlenhorst* Rohde, Bischoff & Tiedje = 44 z l, w  
Z Hyg in press
- Salmonella vleuten* = 44 f, g -
- \* *Salmonella vrindaban* = 45 a c, n, x (45<sub>1</sub>, 45<sub>3</sub>)
- Salmonella wangala* = 1, 9, 12 z<sub>1</sub>, z<sub>21</sub> 1, 7
- Salmonella wedding* = 28 c c, n, z<sub>1</sub>
- Salmonella wildwood* = (3), (15), 34 c, h l, w
- \* *Salmonella* 1, 9, 12 z<sub>23</sub> 1, 7
- \* *Salmonella* 3, 15 g, m, s, t
- Salmonella* 1, 3, 19 c, h l, w
- Salmonella* 13, 22 g m c, n, z<sub>1</sub>
- \* *Salmonella* 13, 22 g, m, t -
- Salmonella* 21 c c, n, x
- Salmonella* 45 g, m - (45<sub>1</sub> 45<sub>1</sub>)
- \* *Salmonella* 51 - 1, 7

Die Sonderstellung des *sub genus* II kommt auch bei der Verteilung der H Antigene zum Ausdruck, da besonders häufig folgende H Antigen komplexe vertreten sind diphasische G Komplexe wie g,m,s,t<sub>z12</sub> oder g,m,s,t<sub>z39</sub> oder f,g,t<sub>e,n,x,z15</sub> etc., die H-Antigene z, z<sub>39</sub> und z<sub>12</sub> sowie die 2 Phasen 1, 5, 7 und e,n,x,z<sub>15</sub>. Bisher sind die H Antigene z<sub>39</sub> und z<sub>12</sub> sowie 1,5,7 und e,n,x,z<sub>15</sub> noch nicht bei *species* des *sub-genus* I gefunden *S. lusingen* = 48 a 1,5,7 ist eine intermediäre *species* und konnte zum *sub-genus* II gerechnet werden, obwohl sie Gelatine nicht verflüssigt.

Die 2 Phasen 1, 5, 7 und e,n,x,z<sub>15</sub> kommen wiederholt im *sub-genus* III (Arizona) vor, doch sind die H-Antigene z<sub>39</sub> und z<sub>12</sub> bisher noch nicht in diesem *sub genus* gefunden worden. Die 3 *sub genera* haben also eine charakteristische Antigenverteilung, die weitere Beachtung verdient.

So ist z.B. damit zu rechnen, dass das H Antigen z, welches komplex gebaut ist und im *sub genus* II sehr häufig vorkommt, von den z Antigenen des *sub genus* I verschieden ist. Das entsprechende gilt auch für andere H Antigene. Während es also mit Rücksicht auf die bakterio-

logischen bestimmt man die e,n,x z<sub>15</sub>-Phase nur nach dem vereinfachten K-W-Schema mit e,n, so übersieht man, dass diese e,n Phasen (= e,n,x, z<sub>15</sub>) im *sub genus* I überhaupt nicht vorkommen. Deshalb wissen wir heute nicht, ob die zahlreichen z-Antigene in den *sub genera* I und II identisch sind oder nicht.

Die Grenzen zwischen den *sub genera* I, II und III sind, wie bei allen biochemischen Gruppen, nicht scharf, sodass es in einigen Fällen willkürlich ist, wohin man eine *species* rechnen will. Während also die *species* Diagnose scharf ist, so ist die *sub genus* Diagnose unscharf. Alle diejenigen *species*, die, wie z.B. *S. schleissheim*, Gelatine verflüssigen, sich aber in den organischen Säuren wie Mitglieder des *sub genus* I verhalten, stehen zwischen I und II. Andere *species*, die Gelatine verflüssigen und Dufelt nicht spalten, stehen zwischen II und III.

In epidemiologischer Hinsicht haben alle weiteren Erfahrungen die frühere Feststellung bestätigt, dass die überwiegende Mehrzahl der *sub-genus* II-species aus Südafrika stammt. Sie sind teils aus Menschen oder Tieren und teils aus Fischmehl, Eiprodukten oder anderen Nahrungsmitteln, die aus Afrika importiert wurden, isoliert.

Da die Zahl der *species* im *sub-genus* I ca. 700 und im *sub genus* II ca. 130 beträgt, so kommen wir auf ca. 1000 *Salmonella species*, wenn wir das *sub-genus* III (Arizona) hinzurechnen. Da ferner die wichtigsten und häufigsten *species* zu den O Gruppen A-E des *sub genus* I gehören, so ist es berechtigt, weitere *species* der *sub genera* II und III nur nach dem vereinfachten K-W-Schema zu diagnostizieren.

## ZUR SEROLOGIE DES SALMONELLA SUB GENUS II

Von

F KAUFFMANN

Eingegangen 15.63

Das *Salmonella sub-genus II* wurde 1960 vom Verfasser in seiner Mitteilung „Two biochemical sub-divisions of the genus *Salmonella*“ aufgestellt und enthielt 55 species. Auf Grund eines Vorschlages von F. Kauffmann & R. Rohde sollen weitere species dieses sub-genus II ab 1963 nur nach dem vereinfachten Kauffmann-White-Schema (K-W-Schema) diagnostiziert werden.

Es erschien deshalb wünschenswert, alle bisher bekannten 131 species dieses sub-genus II in einer Tabelle zusammenzustellen, um einen Überblick über die serologischen Besonderheiten zu erhalten. Über die biochemischen Reaktionen des sub-genus II wurde in einer vorhergehenden Mitteilung des Verfassers „Zur Differentialdiagnose der *Salmonella subgenera I, II und III*“ berichtet, sodass hierauf verwiesen sei.

Die in der folgenden Tabelle nicht aufgeführten ca. 700 species des sub-genus I sind in dem Kapitel „Das Kauffmann-White-Schema“ in dem Buche „The World Problem of Salmonellosis“ (herausgegeben von Dr. E. van Oye) angegeben. Ferner sei auf das Buch des Verfassers „Die Bakteriologie der *Salmonella Species*“ verwiesen sowie auf die beiden Supplemente V und VI zum K-W-Schema.

Bis Ende 1962 waren im ganzen über 830 *Salmonella species* der subgenera I und II bekannt, hiervon gehören 131 zum sub-genus II und ca. 700 zum sub-genus I. Vergleichen wir die O-Gruppen-Verteilung in beiden subgenera, so sehen wir, dass die klassischen O-Gruppen 4–12 beim sub-genus II nur in ca. 30 %, beim sub-genus I aber in ca. 60 % vorkommen. Die höheren O-Gruppen treten also im sub-genus II erheblich häufiger als im sub-genus I auf.

Hierbei ist zu berücksichtigen, dass es sich nur um die Gesamtzahl der species handelt, nicht aber um ihre Häufigkeit im pathologischen Materiale. Berücksichtigen wir diese, so kommen beim sub-genus I hauptsächlich species der O-Gruppen 4–12 vor, während beim sub-genus II die species der höheren Gruppen sehr stark überwiegen.

Gehen wir weiter zum sub-genus III (Arizona) so sind die höheren O-Gruppen noch häufiger als im sub-genus II vertreten, sodass also dieses sub-genus II auch in serologischer Hinsicht eine Zwischenstellung zwischen den subgenera I und III einnimmt.

Species	körper- Antigene	Cassel Antigene	
		1 Phase	2 Phase
westjark	3 10	1 x 9	e n, x
§ alexander	3 10	x	1,5
§ finchlev	3 10	x	e n, x
§ mp la	3 10	x <sub>30</sub>	x <sub>42</sub>
§ winchester	3 10	x <sub>39</sub>	1 7
Gruppe F 2			
§	3 15	g m s t	-
Gruppe F			
montgomery	11	d(a)	d e n x <sub>15</sub>
§ grabouw	11	g m s t	x <sub>30</sub>
§ line ln	11	m t	e n, x
§ huila	11	1 x + 4	e n, x
§ parera	11	x <sub>4</sub> , x <sub>3</sub>	-
Gruppe G 1			
§	13 22	g m t	-
§ rotterdam	1 13, 22	g t	1 5
§ el ft n	13, 22	x <sub>49</sub>	1 5
§ goodwood	13, 22	x <sub>49</sub>	e, n x
Gruppe G 2			
§ aeres	1 13 23	b	x <sub>40</sub>
§ luanshya	13 23	g s (t)	-
§ gojenberg	1 13 23	g t	1 5
§ katesgrove	1 13 23	m t	1 5
§ worcester	1 13, 23	m t	e n, x
§ nachshonim	1 13, 23	x	1 5
§ stevenage	1 13, 23	x <sub>15</sub>	1 7
Gruppe I			
§ tellville	16	e n, x	1 7
§ mobeni	16	g m s t	-
§ mersey side	16	g t	1 5
§ rowbarion	16	m t	-
§ ha id n	16	x <sub>4</sub> , x <sub>3</sub>	-
§ jacksonville	16	x <sub>49</sub>	-
§ w oodstock	16	x <sub>40</sub>	1 (5) 7
§ elsie rivier	16	x <sub>40</sub>	1 6
Gruppe J			
§ hillbrew	17	b	e n, x, x <sub>15</sub>
§ verity	17	e n, x, x <sub>15</sub>	1 6
§ bleat n	17	f g t	(e n, x, x <sub>15</sub> )
Gruppe k			
§ zeist	18	x <sub>10</sub>	x <sub>4</sub>
§ bel ha	18	x <sub>10</sub>	-

## KAUFFMANN-WHITE-SCHEMA

## Sub genus II

Species	Körper- Antigene	Geissel Antigene	
		1 Phase	2 Phase

Gruppe B			
S sofia	4 12	b	~
S sofia var 27	4 12 27	b	~
S makumira	4 12	enx	17
S caledon	4 12	gm	enx
S bechuana	4 12 27	gt	~
S kilwa	4 12	lw	enx
S nordenham	1 4 12 27	z	enx
S durhanyille	4 12	z39	157

Gruppe C 1			
S calvinia	6 7	a	z4
S bloemfontein	6 7	b	z49
S heilbron	6 7	1 z93	15
S tosamanga	6 7	z	15
S haccngo	6 7	z39	z49
S gilbert	6 7	z51	17
S sullivan	6 7	z19	17

Gruppe C 2			
S tular	6 8	a	z99
S haragwanath	6 8	mt	15
S germiston	6 8	mt	enx

Gruppe D 1			
S mjimwema	1 9 12	b	enx
S blakenese	1 9 12	b	z6
S zuerich	1 9 12	c	z39
S lindrick	9 12	enx	157
S lindrick var 17	9 12	enx	17
S kuilsrivier	9 12	gmxt	enx
S manica	1 9 12	gmxt	z49
S neasden	9 12	gst	enx
S hamburg	1 9 12	gt	
S dar es salaam	1 9 12	lw	enx
S stellenbosch	1 9 12	#	17
S angola	1 9 12	z	z6
S hueningen	9 12	z	z39
S canastel	9 12	z9	15
S	1 9 12	z99	17

Gruppe D 2			
S lundby	(9) 46	b	enx
S haarlem	(9) 46	z	enx

Gruppe F 1			
S chudleigh	3 10	enx	17
S islington	3 10	gt	~
S fuhsbuettel	3 10	lv	z6

Species	Körper- Antigene	Cellul Antigene	
		1 Phase	2 Phase
Gruppe W			
W vridaban	43	a	e n, x
S ejeda	45	a	x <sub>10</sub>
S bremen	45	g m s t	e n, x
S windhoek	45	g t	1,5
W perinet	45	m t	e n, x, x <sub>15</sub>
S klapmuts	45	z	x <sub>20</sub>
Gruppe V			
S bilthoven	47	a	—
S phoenix	47	b	1,5
S quimbamba	47	d	x <sub>20</sub>
S chersipa	47	z	x <sub>2</sub>
Gruppe Y			
S hammonia	48	e n, x, x <sub>15</sub>	x <sub>6</sub>
S sakaraha	48	k	x <sub>20</sub>
S ngozi	48	x <sub>10</sub>	1,5
Gruppe Z			
S krugersdorp	50	e n, x	1,7
S wassenaar	50	g (p) x <sub>21</sub>	—
S greensile	50	z	e n, x
S bonaire	50	x <sub>4</sub> , x <sub>25</sub>	—
S hooggraven	50	x <sub>10</sub>	x <sub>6</sub> , x <sub>14</sub>
Gruppe 51			
S	51	—	1,7
Gruppe 52			
S l hatsi	52	—	1,5, 7
Gruppe 53			
S midhurst	53	1, 2, 9	x <sub>20</sub>
S humber	53	z <sub>1</sub> , x <sub>24</sub>	—
Gruppe 55			
S tranoroa	55	k	x <sub>20</sub>
Gruppe 56			
S artis	56	ll	—
Gruppe 57			
S ycaroo	57	x <sub>29</sub>	x <sub>14</sub>
S manombo	57	x <sub>29</sub>	e n, x, x <sub>15</sub>



Species	Körper Antigene	Geissel Antigene	
		1 Phase	2 Phase
Gruppe L			
S soesterberg	21	z <sub>1</sub> z <sub>23</sub>	
S gwaai	21	z <sub>4</sub> z <sub>24</sub>	—
S wandsbek	21	z <sub>10</sub>	z <sub>6</sub>
Gruppe M			
S kaltenhausen	28	b	z <sub>6</sub>
S cerea	28	z	z <sub>39</sub>
Gruppe P			
S carletonville	38	d	—
S foulpointe	38	g t	—
Gruppe Q			
S mondeor	39	l z <sub>24</sub>	e n v
Gruppe R			
S springs	40	a	z <sub>39</sub>
S suariz	1 40	■	e n v z <sub>15</sub>
■ alsterdorf	1 40	g m t	—
S bokshurg	40	g s	e n v z <sub>10</sub>
S bulawayo	1 40	z	1 5
■ sachsenwald	40	z <sub>4</sub> z <sub>23</sub>	—
S degania	40	z <sub>4</sub> z <sub>24</sub>	—
S bern	1 40	z <sub>4</sub> z <sub>12</sub>	—
S sandran	1 40	z <sub>33</sub>	e n v z <sub>17</sub>
S grunty	1 40	z <sub>39</sub>	1 6
Gruppe S			
■ lethe	41	g t	
S negev	41	z <sub>10</sub>	1 2
S lichtenberg	41	z <sub>10</sub>	z <sub>6</sub>
Gruppe T			
S chinovum	42	b	1 5
■ uphill	42	b	e n v z <sub>1</sub>
S fremantle	42	(f) g t	
S portbech	42	l v	e n v z <sub>15</sub>
S nairobi	42	r	
S detroit	42	z	1 5
S rand	42	■	e n v z <sub>15</sub>
Gruppe U			
S houten	43	z <sub>4</sub> z <sub>1</sub>	
S tuindorp	43	z <sub>4</sub> z	
S bunnik	43	z <sub>4</sub>	[1 5 7]

Species	körper- Antigene	Cellul Antigene	
		1 Phase	2 Phase
Gruppe W			
S vrindaban	45	a	en x
ejeda	45	a	z <sub>10</sub>
S bremen	45	g m s t	en x
S windhoek	45	g t	1,5
perinet	45	m t	en x z <sub>15</sub>
S klappmuts	45	z	z <sub>30</sub>
Gruppe X			
S bilthoven	47	a	-
S phoenix	47	b	1,5
S quimbamba	47	d	z <sub>30</sub>
S chersina	47	z	z <sub>6</sub>
Gruppe Y			
S hammonia	48	en x z <sub>15</sub>	z <sub>6</sub>
sakaraha	48	k	z <sub>30</sub>
S ngizi	48	z <sub>10</sub>	1,5
Gruppe Z			
S krugersdorp	50	en x	1,7
S wassenbaat	50	g (p), z <sub>15</sub>	-
S greenside	50	z	en x
S bonaire	50	z <sub>1</sub> z <sub>30</sub>	-
S hoggraven	50	z <sub>10</sub>	z <sub>6</sub> z <sub>15</sub>
Gruppe 51			
S	51	-	1,7
Gruppe 52			
S lobatsi	52		1,5,7
Gruppe 53			
S midhurst	53	1 z <sub>30</sub>	z <sub>30</sub>
S humler	53	z <sub>1</sub> z <sub>30</sub>	-
Gruppe 55			
S tranoroa	55	k	z <sub>30</sub>
Gruppe 56			
S artis	56	b	-
Gruppe 57			
S Icaro	57	z <sub>30</sub>	z <sub>15</sub>
S manombo	57	z <sub>30</sub>	en x z <sub>15</sub>

Species	Körper Antigene	Geißel Antigene	
		1 Phase	2 Phase
Gruppe 58			
S basel	58	1 2 13 204	1 5
Gruppe 59			
S bettoky	59	k	(z)
Gruppe 60			
S luton	60	z	en v

### ZUSAMMENFASSUNG

Es werden die Antigenformeln der bisher bekannten 131 *Salmonella species* des sub-genus II in einer Tabelle zusammengestellt und mit den ca 700 *species* des sub-genus I verglichen

Zu den O-Gruppen A–C gehören nur ca 30 % der *species* des sub genus II, über ca 60 % derjenigen des sub genus I. Beim sub genus II kommen also überwiegend die höheren O-Gruppen vor, sodass dieses sub-genus auch in serologischer Hinsicht eine Zwischenstellung zwischen den sub genera I und III (Arizona) einnimmt

Auch die H-Antigen-Verteilung innerhalb der 3 sub-genera ist deutlich verschieden, sodass diese einen charakteristischen Antigenaufbau besitzen

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## THE EFFECT OF THALIDOMIDE ON THE GROWTH CURVE OF A RIBOFLAVIN DEPENDENT MICROBE

By

TORRE MIDTVEDT

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During the last year, numerous reports have appeared which indicate that the administration of thalidomide to women during early pregnancy may be followed by the birth of a malformed child. A brief survey of the problems concerned has recently been given by Schrader & Stevers (1962). In an attempt to explain the teratogenicity of thalidomide, a very interesting theory has been proposed by Leck & Miller (1962). Supported by clinical observations they point out that there are several similarities between the effect of thalidomide on human beings and those of riboflavin-deficiency in rats. No animal experiments have as yet been published, however, which elucidate this problem.

It has been the purpose of the present investigation to study the effect of thalidomide on the growth of riboflavin dependent microbes. A brief report concerning some of the results have previously appeared in Norwegian (Midtvedt 1963 a, Midtvedt 1963 b).

### MATERIALS AND METHODS

#### Bacterial Strains

As the main test strain a

As the main test strain a

#### Culture Technique

Growth curve analyses were performed in

Growth curve analyses were performed in

Species	Körper Antigene	Geißel Antigene	
		I Phase	II Phase
Gruppe 58			
<i>S. basel</i>	58	1 2 3 4	1 5
Gruppe 59			
<i>S. bettovi</i>	59	k	(z)
Gruppe 60			
<i>S. lutea</i>	60	z	en v

## ZUSAMMENFASSUNG

Es werden die Antigenformeln der bisher bekannten 131 *Salmonella* species des sub genus II in einer Tabelle zusammengestellt und mit den ca. 700 species des sub genus I verglichen.

Zu den O Gruppen A-E gehören nur ca. 30 % der species des sub genus II, aber ca. 60 % derjenigen des sub genus I. Beim sub genus II kommen also überwiegend die höheren O Gruppen vor, sodass dieses sub-genus auch in serologischer Hinsicht eine Zwischenstellung zwischen den sub-genera I und III (Arizona) einnimmt.

Auch die H-Antigen-Verteilung innerhalb der 3 sub genera ist deutlich verschieden, sodass diese einen charakteristischen Antigenaufbau besitzen.

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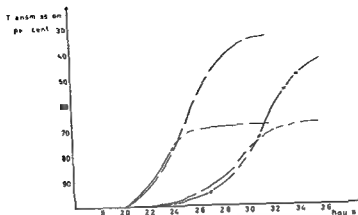


Fig. 1

Rate of growth of *Lactobacillus delbrueckii* in a medium containing varying concentrations of Riboflavin and with/without a concentration of Thalidomide of 100 mcg/ml

— — — — —	Riboflavin 0.0010 mcg/ml	Thalidomide 0 mcg/ml
- - - - -	Riboflavin 0.0010 mcg/ml	Thalidomide 100 mcg/ml
— + — + —	Riboflavin 0.0150 mcg/ml	Thalidomide 0 mcg/ml
- - - - -	Riboflavin 0.0150 mcg/ml	Thalidomide 100 mcg/ml

Thalidomide was suspended in sterile distilled water and added to the medium.

Ug (Zde 1962)

cent is destroyed by auto-

### Photometry

The growth rate was determined by turbidimetric readings in a Beckman Model C colorimeter with green filter. This filter gives maximum transmittance at 624 mμ.

### Chemicals

Niacin and biotin were obtained commercially through Norsk Medisinaldepot. Riboflavin was obtained from E. Merck AG Darmstadt, Germany. Thalidomide was kindly supplied by Norsk Astra A/S Oslo.

## RESULTS

The first problem which was attacked was the influence of thalido-

on the growth of *L. delbrueckii*.

Table 1

It has been recorded which demonstrate a significant change in the growth response of *Lactobacillus delbrueckii* as the results of an addition of thalidomide. The influence of thalidomide becomes apparent when the data are arranged in a growth diagram as shown in Fig. 1. Apparently the lag phase is approximately 6 hours shorter when thalidomide is present than in the control without. In contrast to this no significant change can be demonstrated in the other growth

TABLE 1  
*Rate of Growth of Lactobacillus delbrückii in a Medium Containing Varying Concentrations of Riboflavin  
 Each Concentration of Riboflavin Is Tested with and without a Constant Amount of Thiamide*

Riboflavin in cc ml	Thiamide in mg ml	Inoculum in ml	Hours																41	48
			18	21	24	27	30	33	36	39	42	45	48	51	54	57	60	63		
0.0	0.0	0.0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
0.0	0.0	0.2	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
0.0	100	0.2	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
0.0025	0.0	0.2	100	98	98	97	96	95	95	95	95	95	95	95	95	95	95	95	95	95
0.0025	100	0.2	100	100	97	96	88	83	80	76	74	74	74	74	74	74	74	74	75	71
0.0050	0.0	0.2	100	99	98	98	98	97	94	91	88	84	84	84	84	84	84	84	84	84
0.0050	100	0.2	100	100	98	85	78	72	71	71	69	69	69	69	69	69	69	69	69	69
0.0075	0.0	0.2	100	100	99	98	95	95	95	94	93	88	83	79	70	66	64	60	59	59
0.0075	100	0.2	100	99	99	81	75	67	62	61	60	59	59	59	59	59	59	59	59	59
0.01	0.0	0.2	100	100	98	98	98	98	96	95	93	88	81	76	71	64	60	57	54	51
0.01	100	0.2	100	100	100	89	79	70	61	57	53	52	50	50	50	50	50	50	50	50
0.015	0.0	0.2	100	100	100	99	99	97	95	95	93	87	81	74	62	56	50	44	36	35
0.015	100	0.2	100	100	100	88	79	69	58	48	41	40	36	36	35	35	35	35	35	35
0.02	0.0	0.2	100	100	100	100	100	99	93	88	81	71	71	54	41	34	30	25	25	25
0.02	100	0.2	100	99	89	68	52	44	38	33	29	27	26	26	26	26	25	25	25	25
0.03	0.0	0.2	100	100	100	98	98	97	94	89	83	75	75	56	45	36	31	18	17	17
0.03	100	0.2	100	98	91	67	53	45	39	31	28	24	24	22	19	19	19	18	18	18

Each figure represents the mean of the reading of two different samples, and the results are given as per cent transmittancy

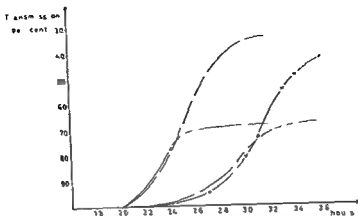


Fig. 1

Rate of growth of *Lactobacillus delbrueckii* in a medium containing varying concentrations of Riboflavin and with/without a concentration of Thalidomide of 100 mcg/ml

— — — — —	—	Riboflavin 0.0030 mcg/ml	Thalidomide 0 mcg/ml
- - - - -	-	Riboflavin 0.0030 mcg/ml	Thalidomide 100 mcg/ml
+ + + + +	+	Riboflavin 0.0150 mcg/ml	Thalidomide 0 mcg/ml
- - - - -	-	Riboflavin 0.0150 mcg/ml	Thalidomide 100 mcg/ml

Thalidomide —

It is known that only a small part—about 5 per cent—is destroyed by autoclaving (Fide 1962)

### Photometry

The growth rate was determined by turbidimetric readings in a Beckman Model C colorimeter with green filter. This filter gives maximum transmittance at 524 mμ.

### Chemicals

Niacin and biotin were obtained commercially through Norsk Medicinaldepot. Riboflavin was obtained from F. Merck, AG Darmstadt, Germany. Thalidomide was kindly supplied by Norsk Astra A/S Oslo.

## RESULTS

The first problem which was attacked was the influence of thalidomide on the various phases of the growth curve of a riboflavin requiring microbe in which the growth was limited by riboflavin. In Table 1 some

results

of an experiment showing the influence of thalidomide becomes apparent when the data are arranged in a growth diagram as shown in Fig. 1. Apparently the lag phase is approximately 6 hours shorter when thalidomide is present than in the control without. In contrast to this, no significant change can be demonstrated in the other growth



TABLE 2  
Rate of growth of *Lactobacillus delbrückii* in a Media Containing Constant Concentration of Riboflavin and Varying Concentration of Thiamide

Riboflavin in mc. ml	Thiamide in mc. ml	Inoculum in mc. ml	Hours												■	37
			18	20	22	23	24	25	26	27	28	29	30	31		
0.0	0.0	0.0	100	100	100	100	100	100	100	100	100	100	100	100	100	100
0.0	0.0	0.0	100	100	100		98	97				94			93	93
0.0	100	0.2	100	100			99	98				97			97	97
0.01	0.0	0.2	100	100	100		98	96	92	87	78	70	64		64	55
0.01	200	0.2	100	95	86	79	69	60	45	31	51	52	52		52	51
0.01	100	0.2	100	96	87	78	68	61	57	56	55	54	53		53	52
0.01	50	0.2	100	96	89	81	73	64	59	55	54	54	53		53	51
0.01	10	0.2	100	97	93	87	77	67	60	56	54	53	52		52	52
0.01	5	0.2	100	98	95	90	84	74	66	61	58	56	55		55	53
0.01	1	0.2	100	98	98	96	90	87	82	72	64	60	59		59	54
0.01	0.5	0.2	100	100	100	96	93	90	83	74	65	60	58		58	54
0.01	0.10	0.2	100	100	100	97	97	97	91	84	77	68	61		61	56
0.01	0.05	0.2	100	100	100	98	97	96	91	84	73	66	63		63	55

Each figure recorded is the mean of the reading of two different samples and the results are given as per cent transmittancy

TABLE 3  
Rate of growth of *Lactobacillus arabinosus* in Media Containing Varying Concentration of Viacin and Thiamide

Viacin in mc. ml	Thiamide in mc. ml	Inoculum in ml	Hours												27	29
			15	16	17	18	19	20	21	22	23	24	25	26		
0.0	0.0	0.0	100	100	100	100	100	100	100	100	100	100	100	100	100	100
0.0	0.0	0.2	100	100	100	100	100	100	100	94		90	88	88	88	88
0.0	100	0.2	100	100	100	100	100	100	100	96		91	89	88	88	88
0.02	0.0	0.2	100	93	96	90	83	74	58	45	34	28	25	23	21	20
0.02	100	0.2	100	99	95	88	81	70	53	40	31	26	23	22	21	19

Each figure recorded is the mean of the reading of two different samples and the results are given as per cent transmittancy

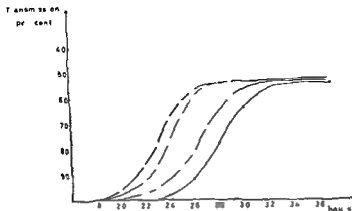


Fig. 2

Rate of growth of *Lactobacillus delbrueckii* in a media containing constant concentration of Riboflavin and varying concentrations of Thalidomide

— + — + — + — + —	Riboflavin	0.01 mcg/ml	Thalidomide	0 mcg/ml
— — — — —	Riboflavin	0.01 mcg/ml	Thalidomide	1 mcg/ml
— — — — —	Riboflavin	0.01 mcg/ml	Thalidomide	10 mcg/ml
— — — — —	Riboflavin	0.01 mcg/ml	Thalidomide	100 mcg/ml

TABLE 4

Rate of Growth of *Lactobacillus arabinosus* in a Medium Containing Varying Concentrations of Biotin and Thalidomide

Biotin in mcg/ml	Thalidomide in mcg/ml	Inoculum in mcg/ml	Hours						
			16	19	23	24	25	29	35
0.0	0.0	0.2	100	100	100	100	100	100	100
0.0	0.0	0.0	100	100	98	96	90	90	90
0.0	100	0.2	100	100	97	95	89	89	89
0.0003	0.0	0.2	100	98	85	78	69	48	47
0.0003	100	0.2	100	99	84	77	66	46	45

Each figure recorded is the mean of the reading of two different samples and the results are given as per cent transmittance.

phases. Furthermore riboflavin is in each instance acting as the growth limiting factor.

In a following series of experiments the quantity of thalidomide necessary in order to reduce the lag phase was studied. From the data presented in Table 2 it may be seen that the shortening of the lag phase is indeed dependent on the concentration of thalidomide. The shortest lag phase is found with a concentration of 100 mcg per ml. Higher concentrations give no further changes in the pattern of the growth curve than that obtained with 100 mcg per ml. When the concentration of thalidomide is reduced below 100 mcg per ml the lag phase is steadily increased. At the concentration of 0.1 mcg per ml no effect can be observed on the lag phase which is then identical with that found in media



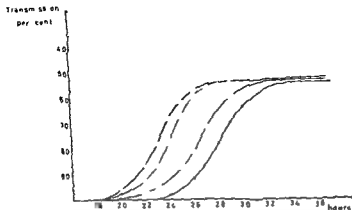


Fig 2

Rate of growth of *Lactobacillus delbrueckii* in a media containing constant concentration of Riboflavin and varying concentrations of Thalidomide

— + — + — + —	Riboflavin	0.01 mcg/ml	Thalidomide	0 mcg/ml
— — — — — — —	Riboflavin	0.01 mcg/ml	Thalidomide	1 mcg/ml
— · — · — · — · —	Riboflavin	0.01 mcg/ml	Thalidomide	10 mcg/ml
— · — · — · — · —	Riboflavin	0.01 mcg/ml	Thalidomide	100 mcg/ml

TABLE 4

Rate of Growth of *Lactobacillus arabinosus* in a Medium Containing Varying Concentrations of Biotin and Thalidomide

Biotin in mcg/ml	Thalidomide in mcg/ml	Inoculum in mcg/ml	Hours						
			18	19	23	24	25	29	30
0.0	0.0	0.2	100	100	100	100	100	100	100
0.0	0.0	0.0	100	100	98	96	90	90	90
0.0	100	0.2	100	100	97	95	89	88	89
0.00003	0.0	0.2	100	98	85	78	69	48	47
0.00003	100	0.2	100	99	84	77	66	46	45

Each figure recorded is the mean of the reading of two different samples and the results are given as per cent transmittancy

phases. Furthermore, riboflavin is in each instance acting as the growth limiting factor.

In a following series of experiments, the quantity of thalidomide necessary in order to reduce the lag phase was studied. From the data presented in Table 2 it may be seen that the shortening of the lag phase is indeed dependent on the concentration of thalidomide. The shortest lag phase is found with a concentration of 100 mcg per ml. Higher concentrations give no further changes in the pattern of the growth curve than that obtained with 100 mcg per ml. When the concentration of thalidomide is reduced below 100 mcg per ml, the lag phase is steadily increased. At the concentration of 0.1 mcg per ml, no effect can be observed on the lag phase which is then identical with that found in media

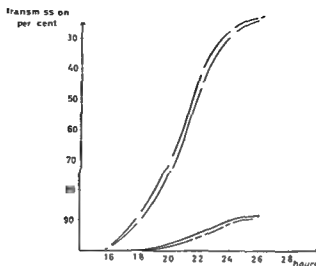


Fig 3

Rate of growth of *Lactobacillus arabinosus* in a medium containing varying concentrations of Niacin and Thalidomide

— + — + — + —	= Niacin 0	mcg/ml, Thalidomide 0	mcg/ml
— — — — —	= Niacin 0	mcg/ml, Thalidomide 100	mcg/ml
— — — — —	= Niacin 0.02	mcg/ml, Thalidomide 100	mcg/ml
— — — — —	= Niacin 0.02	mcg/ml, Thalidomide 0	mcg/ml

without thalidomide. This thalidomide effect has also been illustrated by the graphs presented in Fig 2.

The next problem to be studied was that of the specificity of this thalidomide effect. Growth experiments in similar technique were performed with the vitamins niacin and biotin as recorded in Table 3 and Table 4. From the data presented no significant effect from thalidomide can be demonstrated on the growth curves in microbes in which the growth is limited by either niacin or biotin. The thalidomide insensitive niacin assays have been illustrated in the graphs of Figure 3.

## DISCUSSION

When the riboflavin dependent *Lactobacillus delbrueckii* is growing with riboflavin as the limiting factor in the presence of thalidomide, the lag phase is significantly shortened. This decrease in time is independent of the concentration of riboflavin, and the final production of bacteria is still determined—limited—by the riboflavin available. The total synthesis of bacteria is accomplished approximately 6 hours earlier when thalidomide is present than when absent.

A concentration of 100 mcg per ml of thalidomide has a maximum influence on the lag phase under the conditions of the assay. Higher concentrations do not further change the growth curves. In this connection it should be remarked that the concentration of 100 mcg per ml corresponds to the maximal amount of thalidomide actually dissolved under these conditions.

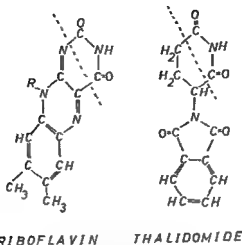


Fig. 4

Formulae of riboflavin and thalidomide. The  $\text{CO-NH-CO}$  sequence is shown at the top of each formula.

Experiments with other vitamins as growth limiting factors may be taken to indicate a specificity in the relation between thalidomide and riboflavin even if too definite conclusions would be premature at the present stage.

The interpretation of these findings is as yet far from clear. A comparison of riboflavin and thalidomide, such as drawn in Fig. 4, indicates that both have the sequence— $\text{CO-NH-CO}$ —. It has been assumed (Leck & Miller 1962) that the  $\text{NH}$  group is one point at which the coenzymes which contain riboflavin combine with their apo-enzymes. Thus, it seems conceivable that thalidomide exerts its toxic effects, in the embryo as well as in adult, either by competing with riboflavin-containing coenzymes for attachment to their apoenzymes or by inactivation of the vitamin by combining with it. In either case, the effect would be that of a vitamin deficiency. In the biological systems used in the present investigation, such a deficiency should have resulted in signs which indicate a retardation of the growth, i.e. lengthening of the lag phase, a flat logarithmic phase, and probably a decrease in the amount of cells synthesized. Instead of this, we observed an acceleration in the start of growth when thalidomide is added.

It might well be that thalidomide, on account of its similarities with

in any case, the results presented indicate new possibilities for the study of the effect of thalidomide on the metabolism, since many well known microbial systems are at hand. Such systems include the elaborate instruments of microbial genetics.

## SUMMARY

The effect of thalidomide on the growth of some vitamin requiring microbes has been examined

No effect on the growth curve is found when niacin or biotin is used as the growth limiting factor

When riboflavin is the limiting factor, thalidomide changes the growth curves This effect is concerned with the lag phase which is significantly shortened The effect is dependent of the concentration of thalidomide, and independent of the concentration of riboflavin

The experiments demonstrate a new, reproducible biological effect of thalidomide in addition to the known sedative effect and the probable teratogenic effect in man

## REFERENCES

- 1 *Eide L* Personal communication 1962
- 2 *Leck I M & Miller E I M* Incidence of malformations since the introduction of thalidomide *British Medical Journal* 2 16 1962
- 3 *Multtiedt T* Letter to the editor *Tidsskrift for den Norske Lægeforening* 83 48 1963
- 4 *Multtiedt T* Effekten av thalidomide på veksten av en riboflavinhengig mikrobe *Tidsskrift for den Norske Lægeforening* 1963 (In press)
- 5 *■ Schrader & Siegers* Thalidomide und Missbildungen *Therapeutische Briefe* Grunenthal 8 33 1962

## TUBERCULIN PRODUCTION

### 1 Yield of Tuberculoprotein from Various Media

By

MOGENS MAGNUSSEN, HYUN KYU KIM and  
M. WEIS BENTZON

Received 12/63

The methods used in the sundry laboratories for preparing tuberculin (Seibert 1934, Seibert & Glenn 1941, Jensen 1938, Lind 1947, 1948, McIntosh & Konst 1947, Breley & Lamensans 1951, Lamensans, Grabar & Breley 1951, Green 1953, Svenkerud 1955, Magnusson & Bentzon 1958, Desbordes 1958 Ministry Hlth Welf, Jap 1961) vary in many respects, there are differences in the media and strains, the incubation time for the cultures, the method of concentration, and the purification procedure, etc. It is also obvious from the literature that the yield of tuberculin varies and possibly there are differences in the specificity of the tuberculin produced by the various methods. However, it is not possible to attribute the variation in the yield to definite factors in the production method since so many aspects vary simultaneously. In order, therefore, to obtain a better basis for selection of a rational production method, a series of experiments were carried out to study the variations in tuberculin yield in relation to the composition of the medium, the strain, and the incubation period. The experiments also included comparison between the tuberculin content of culture filtrates and the amount of tuberculin which can be extracted from the bacterial cells. The results of these experiments will be published in a series of articles.<sup>1</sup>

In the present work, comparison is made between seven different media, four of which are those commonly employed, viz. Sauton (Sauton 1912), Long (Long & Seibert 1926), Dorset as modified by the Bureau of Animal Industry, USA (Dorset 1934), and glycerol broth. Three others were included because previous experiments with them had shown high yields of tuberculoprotein, viz. Lind bill medium (Lind 1948), glucose medium, and P medium. Three strains of *Mycobacterium tuberculosis* were used for the comparison, and the incubation period

<sup>1</sup> The experimental work was performed in 1955.



## SUMMARY

The effect of thalidomide on the growth of some vitamin requiring microbes has been examined

No effect on the growth curve is found when niacin or biotin is used as the growth limiting factor

When riboflavin is the limiting factor, thalidomide changes the growth curves. This effect is concerned with the lag phase which is significantly shortened. The effect is dependent of the concentration of thalidomide, and independent of the concentration of riboflavin

The experiments demonstrate a new, reproducible biological effect of thalidomide in addition to the known sedative effect and the probable teratogenic effect in man

## REFERENCES

- 1 *Fide L* Personal communication 1962
- 2 *Leck I W & Miller F L W* Incidence of malformations since the introduction of thalidomide *British Medical Journal* 2 16 1962
- 3 *Multvedt T* Letter to the editor *Tidsskrift for den Norske Lægeforening* 83 48 1963
- 4 *Multvedt T* Effekten av thalidomide på veksten av en riboflavinavhengig mikrobe *Tidsskrift for den Norske Lægeforening* 1963 (in press)
- 5 *Schrader & Sievers* Thalidomide und Missbildungen *Therapeutische Briefe* Grunenthal 8 33 1962

**Synthetic media** The composition of the synthetic media is shown in Table 1. The media were prepared by dissolving the salts in a small quantity of the water, if necessary by heating. After adding the rest of the water and the glycerol the pH was adjusted in the Sauton and glucose media by adding concentrated  $\text{NH}_3$ .

Distribution was made of 180 ml amounts into small flasks which were sterilized by autoclaving (20 minutes at  $120^\circ\text{C}$ ). In the case of the glucose medium, the glucose was sterilized separately as an 1% per cent (w/v) solution and added to the medium aseptically.\*

The Lind bII medium was turbid when prepared and a small precipitate was found in each flask after sterilization, this disappeared during growth of the cultures. A small constant precipitate formed during autoclaving of the Dorset medium.

### Strains

Three virulent strains of *Mycobacterium tuberculosis* were used. One strain T3487 was freshly isolated from a patient at the time of the experiment. The two others E9636 and U1921 were old laboratory strains isolated from patients more than 10 years previously.

Two cultures of each strain grown on 180 ml Sauton medium were used for 16 days old by the medium. The membrane with a thin, regular

membrane covered the surface of the medium.

### Inoculation and Incubation

A pellicle with a diameter of about 10 mm was used as inoculum for each flask. The cultures were incubated at  $38^\circ\text{C}$  and one or two flasks of each medium were sterilized by heating in streaming steam for one hour after 8 days 2 3 4 5 6 7, 8

TABLE 1  
Composition of Synthetic Media

	Sauton	Long	Dorset	Lind bII	Glucose	P medium
Asparagine	g 4	5	14	8	6	12
Citric acid	g 2			0.4*	2	2
Potassium orthophosphate di H	g 0.5	3	18	2.2†	15	2
Magnesium sulphate	g 0.5	1	15	0.5	0.2‡	0.5
Ferric ammonium citrate	g 0.05	0.05		0.05	0.05	0.05
Sodium chloride	g 5					
Sodium carbonate	g 2					
Sodium citrate	g		0.9			
Ferric citrate	g		0.3			
Ammonium citrate	g 5					
Glycerol	ml 50	40	100†	60	30	60
Glucose	g		10		10	
Water distilled	ml 1000			950**		
Water tap	ml	1000	1000*		1000	950
Ammonium hydroxide conc	ml 2.2				1.8	
pH before autoclaving	7.4	7.2	6.9	6.7	6.4	6.8
pH after autoclaving	7.0	6.8	6.8	6.7	6.4	7.0

\* Original 0.2 g

† Original 2.0 g

\*\* Original tap water

‡ If the glucose is heated (sterilized) together with the phosphate toxic compounds are formed (Hong 1937; Dubos & Middlebrook 1947)

varied from one to nine weeks. The protein content of the culture filtrates was measured and is considered in relation to the growth of the bacteria and the pH of the culture filtrates.

The literature contains many reports concerning the yield of tuberculo-protein and tuberculin by cultivation of tubercle bacilli on media of varying composition.

Wong & Weinzirl (1936) modified Long medium by replacing asparagine with ammonium malate and adding glucose. They obtained the same amount of tuberculin with the ammonium malate medium as with the asparagine medium, but in approximately half the time. Later Wong (1937) described another synthetic medium, a glucose-sucrose medium, which gave three times as much protein as could be obtained on Long medium at the end of six weeks. Henley & Le Duc (1939) found that the yield of protein increased when the amount of asparagine and/or glycerol was increased from 4 and 20 g per litre respectively in a synthetic medium which contained the other compounds necessary for the growth. Lind (1948) measured the yield and activity of tuberculin prepared on modifications of Sauton medium, varying the concentration of glycerol and asparagine, and ammonium ferric citrate. The yield of purified tuberculin increased as the asparagine concentration increased, but the activity was reduced simultaneously. Lind modified the medium further by increasing the phosphate concentration (Lind BII medium) and obtained high yield but low activity of the PPD. Asami *et al.* (1953 a, b) measured the protein yield obtained on Sauton and Dorset media and found a higher yield on the latter. Svenkerud (1955) found that the yield of protein increased if sodium hydroxide and acetic acid were added to the Sauton medium. However, the amount necessary to obtain maximum protein yield varied according to the strain used. Gottshall (1962) found that the dry weight of organisms was greater in cultures of tubercle bacilli grown in Henley's synthetic medium (Henley 1929) to which zinc and copper were added and supplemented with calcium, than in those without calcium. The quantity of tuberculo-protein in the culture filtrate was lower in cultures to which calcium had been added than in cultures without calcium.

## MATERIALS AND METHODS

### Media

**Glycerol broth.** The ingredients were—500 g minced veal, 10 g Witte's peptone, 1000 ml tap water. The meat was cut into small pieces and placed in a refrigerator overnight with half the quantity of the fluid. The fluid was pressed out of the meat and the other half of the water. The liquids were mixed and the medium heated and boiled for 5 minutes. Alkalization was carried out with 5 N sodium hydroxide so that after boiling for a further 5 minutes the pH was 7.6 to 7.7. The medium was filtered through filter paper and measured up to volume and finally the glycerol was added. The medium was measured out in 180 ml volumes into small flat bottomed flasks and sterilized by autoclaving for 20 minutes at 120°C. The pH after autoclaving was 7.2.

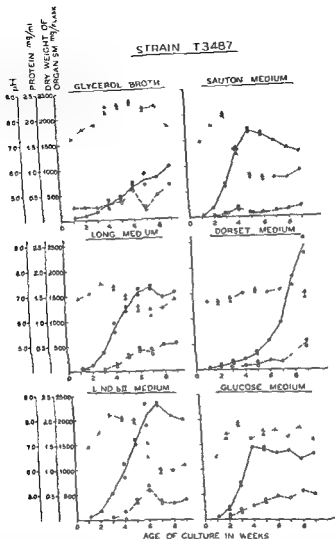


Fig 1

Dry weight of organism (●), pH (Δ) and protein content (○) of culture filtrate of *Mycobacterium tuberculosis* (Strain T3487) cultured on various media in relation to age of culture

The growth rate for the two strains was the same on *Lind bII* medium as on Sauton medium, but the total bacterial crop was higher on the former (about 2300 and 2700 mg). Strain U1921 shows quite strong autolysis on *Lind bII* medium.

While T3487 multiplied with the same speed and almost the same maximum yield of organism on *glucose medium* as on Sauton and Long media, the growth of U1921 was more rapid on *glucose medium* com-

and 9 weeks. After sterilization, the cultures were stored in the refrigerator until time of analysis (1 day to 3 weeks).

### Analysis

**Dry weight.** The content of each flask was poured on to a piece of filter paper (weight determined previously) placed in a Buchner funnel and the culture filtrate removed by means of suction through the paper. The filter paper containing the cells was placed in open metal racks and dried at about 105° C for 24 hours before weighing.

**pH.** The pH of the culture filtrates was determined electrometrically on a sample of the filtrate, using a Radiometer pH Meter 22 with glass electrode.

**Precipitation and estimation of protein.** A sample of 10 ml from each filtrate was precipitated with 11 ml of 50 per cent trichloroacetic acid. After standing in the refrigerator for not less than 24 hours, the precipitates were centrifuged, washed twice with 10 ml of 2 per cent trichloroacetic acid and once with water. Finally the precipitates were dissolved in 1 or 2 ml of 1/10 N sodium hydroxide and the protein content determined by the biuret reaction, using the Weichselbaum biuret reagent (Weichselbaum 1946). The readings were made in a Beckmann spectrophotometer at wave length 540 m $\mu$  using cuvettes containing 0.3 ml (light path 1 cm). The protein content (mg/ml) was calculated from the Beckmann reading by multiplication by 0.9, this factor being determined from Kjeldahl analysis of a reference preparation of tuberculo-protein included in the biuret analysis.

## RESULTS

The results of the experiment are shown in Figs. 1 to 3.

### *Growth of Tubercle Bacilli on the Various Media*

Generally the dry weight of organism increased during the period from 2 to 5-7 weeks. Later autolysis of the bacilli could be seen as the weight of the culture decreased. In a few cases, however, multiplication was so slow that the weight of the cultures increased throughout the whole observation period.

**T3487 and U1921** (Figs. 1 and 2). On *glycerol broth* the growth rate of T3487 and U1921 was slower and the total bacterial crop—about 1000 mg—smaller than on the synthetic media. On *Sauton medium* the total bacterial crop was about 1700 mg for T3487 and about 2000 mg for U1921, in both cases with a culture period of five weeks. After nine weeks the weight of the culture had decreased to about 1300 mg and 1500 mg respectively because of autolysis.

On *Long medium* the growth of the two strains was much the same as on Sauton medium, both as regards yield of bacilli and speed of growth. Autolysis of the bacilli was not so pronounced.

Growth of the two strains on *Dorset medium* was slow. However, the total weight of the bacterial cells after 8 to 9 weeks' growth was large—about 2500 and 2700 mg for the two strains—and it is even possible that these values do not express the maximum yield of bacilli on the medium.<sup>3</sup> The differences between the two flasks of seven- and eight-weeks-old cultures of U1921 were large.

<sup>3</sup> There was no observation after 9 weeks' culture of U1921 because of contamination.

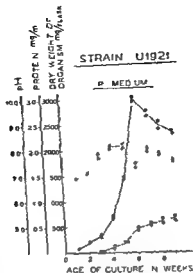


Fig. 2a

Dry weight of organism (●) pH (Δ) and protein content (○) of culture filtrate of *Mycobacterium tuberculosis* (Strain U1921) cultured on a synthetic medium in relation to age of culture

cially in Long Lind hill and glucose media,<sup>4</sup> where the variations between cultures of the same age, and particularly the variations from week to week, were larger than the corresponding variations for the two other strains. Such irregularities in the growth have occurred also in some later experiments with other strains, and in these cases also on Sauton medium. In drawing the figure it has been presumed that there are two different forms of growth, a and b. The one (a) is characterized by large total growth and rapid acidification of the culture filtrate, possibly followed by autolysis. The other (b) is characterized by slower growth, smaller total bacterial crop, and lesser change in pH, probably due to sinking of the pellicle at a different period before sterilization of the culture (see under Discussion). Growth of this strain on the broth was also peculiar. There were no signs of growth from the second to the fifth week, but later slow multiplication was again evident.

Despite this divergence, multiplication of E9656 on the various media shows some of the same characteristics as the other strains. The total bacterial crop was small on the broth (800 mg), not much different in Sauton, Long and glucose media (1700, 2000 and 1800 mg), and largest in Lind hill medium (2400 mg).

However, in contrast to the results with the other two strains, the total crop of bacteria on Dorset medium was relatively small (1500 mg).

<sup>4</sup> Due to a technical mishap it was not possible to record some of the points on the growth curve for Sauton medium.

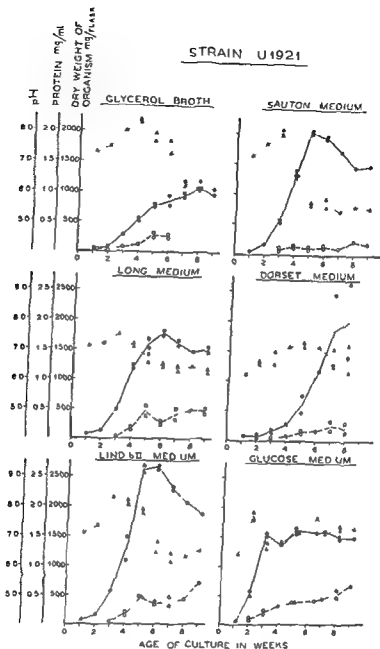


Fig 2

Dry weight of organism (●) pH (Δ) and protein content (○) of culture filtrate of *Mycobacterium tuberculosis* (Strain U1921) cultured on various media in relation to age of culture

pared with the other media, the maximum weight being reached after four weeks. Autolysis of the bacilli on glucose medium was not pronounced.

Only strain U1921 was cultivated on the *P-medium* (Fig 2a). The total bacterial crop (3000 mg) was higher in this medium than in any of the others, and autolysis was fairly strong.

**Strain E9656** The growth of this strain (Fig 3) was irregular, espe-

concentration with the age of the culture, while others show a peak. On Lind bli medium the protein curve for strain E9656 (Fig. 3) shows irregularity similar to that for the growth curve.

The protein curve for T3487 on *broth medium* (Fig. 1) shows a fairly large protein content after one and two weeks. The corresponding bacterial crops were very small. Thus, it would seem probable that the protein found was not produced by the tubercle bacilli but probably originated from the broth. The results of protein analysis on the *broth medium* are therefore not comparable with those on the synthetic media (which do not contain protein) and will not be considered further.

In *Sauton medium* (Figs. 1, 2, 3) the protein content increased very slightly with the age of the culture. However, the largest protein contents (0.2 to 0.3 mg/ml) were small compared with those of the other synthetic media.

In *Long medium* also (Figs. 1, 2, 3) the protein content increased the longer the culture period, but here the maximum protein contents observed were larger (0.6 to 1.4 mg/ml).

The maximum protein contents obtained on *Dorset medium* (Figs. 1, 2, 3) were slightly smaller than those found on *Long medium* (0.3-1.1 mg/ml).

On *Lind bli medium* (Figs. 1, 2, 3) a high protein content was obtained for all three strains (0.7-1.6 mg/ml).

On the *glucose medium* (Figs. 1, 2, 3), the protein content increased regularly with the age of the culture, and the maximum content was fairly high for all three strains (0.6-0.8 mg/ml).

On the *P medium* (Fig. 2a), the highest protein concentration measured was 0.7 mg/ml.

### Measurement of pH

The hydrogen ion concentration was measured after heat sterilization of the cultures, and the pH was found to vary between 5.4 and 8.7. However, the changes in pH evoked by growth of the tubercle bacilli differed from one medium to another. In *Sauton* and *Lind bli* media, the pH first increased to 8.0 or more, later decreased to about 5.5 to 6.0, and sometimes increased again during the latter part of the observation period. In the other media the variations in pH were smaller.

As regards strain F9656 (Fig. 3), the large variations in growth and protein content for *Lind bli* medium were also reflected by the pH measurements. The cultures with large amounts of growth were more acid and had lower protein content than those containing a smaller number of bacteria.

### DISCUSSION

It is a general assumption that tuberculo-protein and tuberculin are released into the culture filtrate of tubercle bacilli by autolysis of the cells (Corper & Cohn 1943, 1944, Green 1953, Svenkerud 1955, Paterson



## STRAIN E9656

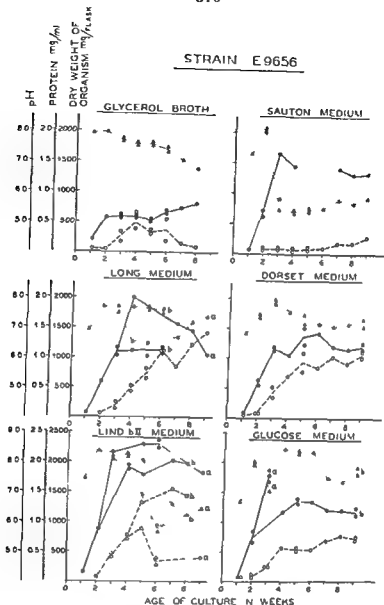


Fig. 3

Dry weight of organism (●), pH (Δ) and protein content (○) of culture filtrate of *Mycobacterium tuberculosis* (Strain E9656) cultured on various media in relation to age of culture

For explanation of a and b curves see text

As compared with T3487 and U1921, the growth rate of E9656 was high, and in particular multiplication of E9656 was not delayed in Dorset medium as it was with the two other strains. Autolysis in the cultures of E9656 could not be estimated with certainty due to the irregular growth.

#### Amount of Protein Present in the Culture Filtrates

The curves for the protein content of the culture filtrates show different patterns. Some of them indicate a regular increase of the protein

concentration with the age of the culture, while others show a peak. On Lind bII medium the protein curve for strain L9656 (Fig. 3) shows irregularity similar to that for the growth curve.

The protein curve for T3487 on *broth medium* (Fig. 1) shows a fairly large protein content after one and two weeks. The corresponding bacterial crops were very small. Thus, it would seem probable that the protein found was not produced by the tubercle bacilli but probably originated from the broth. The results of protein analysis on the *broth medium* are therefore not comparable with those on the synthetic media (which do not contain protein) and will not be considered further.

In *Sauton medium* (Figs. 1, 2, 3) the protein content increased very slightly with the age of the culture. However, the largest protein contents (0.2 to 0.3 mg/ml) were small compared with those of the other synthetic media.

In *Long medium* also (Figs. 1, 2, 3) the protein content increased the longer the culture period, but here the maximum protein contents observed were larger (0.6 to 1.4 mg/ml).

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As regards strain L9656 (Fig. 3), the large variations in growth and protein content for *Lind bII medium* were also reflected by the pH measurements. The cultures with large amounts of growth were more acid and had lower protein content than those containing a smaller number of bacteria.

### DISCUSSION

It is a general assumption that tuberculo-protein and tuberculin are released into the culture filtrate of tubercle bacilli by autolysis of the cells (Corper & Cohn 1943, 1944, Green 1953, Svenkerud 1955, Paterson

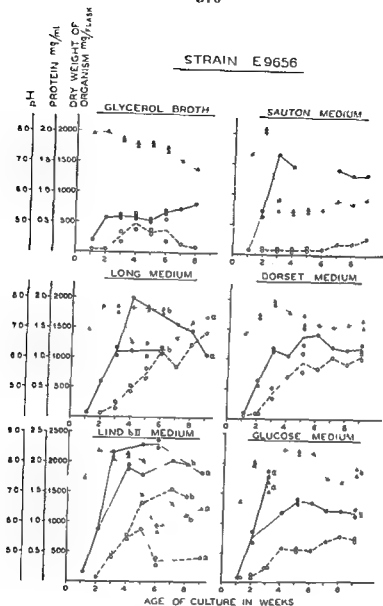


Fig 3

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For explanation of a and b curves see text

As compared with T3487 and U1921, the growth rate of E9656 was high, and in particular multiplication of E9656 was not delayed in Dorset medium as it was with the two other strains. Autolysis in the cultures of E9656 could not be estimated with certainty due to the irregular growth.

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As regards strain E9656 (Fig. 3), the large variations in growth and protein content for *Lind bII medium* were also reflected by the pH measurements. The cultures with large amounts of growth were more acid and had lower protein content than those containing a smaller number of bacteria.

### DISCUSSION

It is a general assumption that tuberculoprotein and tuberculin are released into the culture filtrate of tubercle bacilli by autolysis of the cells (Corper & John 1943, 1944, Green 1953, Svenkerud 1955, Paterson

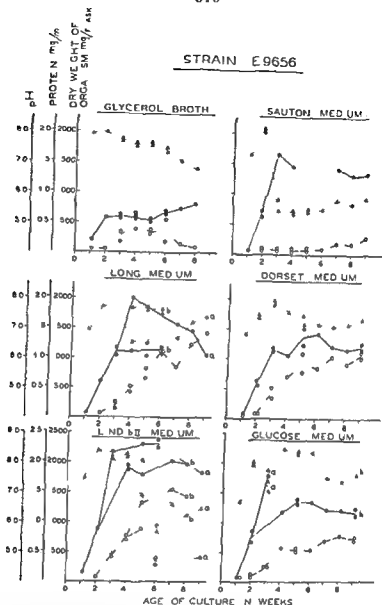


Fig 3

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For explanation of a and b curves see text

As compared with T3487 and U1921 the growth rate of E 9656 was high and in particular multiplication of E 9656 was not delayed in Dorset medium as it was with the two other strains. Autolysis in the cultures of E 9656 could not be estimated with certainty due to the irregular growth.

#### Amount of Protein Present in the Culture Filtrates

The curves for the protein content of the culture filtrates show different patterns. Some of them indicate a regular increase of the protein

strains U1921 and T3487 on Sauton medium Lind's results (1948) indicate a relationship between tuberculin yield and pH of the culture filtrate as regards some strains of *M. tuberculosis*. In addition to the variations in pH, therefore, there would seem to be other factors which exert an influence on the content of tuberculoprotein in the culture filtrate and the tuberculin yield. The observations of Baisden (1951), Paterson *et al* (1958), Dekker & Huilema (1958) and Gottshall (1962) show that zinc, copper, calcium and other trace elements may be deficient in some lots of synthetic media used in tuberculin production, causing the pellicle to sink before it is fully grown, and resulting in a smaller total bacterial crop and a varying yield of tuberculoprotein. Sinking of the pellicle of various strains of *M. tuberculosis* and the BCG strain of *M. bovis* can be prevented by adding zinc and other trace elements to deficient lots of synthetic media (Williston *et al* 1958, 1962, Poulis Pateraki *et al* 1962). Unfortunately, in the present study it was not observed whether the pellicles had sunk in some of the flasks before heat sterilization of the cultures. However, this was observed in a subsequent study with the BCG strain and this is believed to be the explanation of the irregular growth of strain E9656 on some media in the present study. It has been observed previously by others (Paterson *et al* 1958, Dekker & Huilema 1958), that various strains of *M. tuberculosis* show a varying tendency to sink, this is probably the explanation for the regular growth of the two other strains on the same lots of media in the present study.

In the present study there is autolysis of the bacteria as regards strain U1921 (Fig. 2) when cultured for more than five to six weeks on Sauton, Lind III and P media, and simultaneously a certain increase in the protein content of the culture filtrates (Fig. 2). However, the formation of protein is just as rapid, or even more rapid, in the period from 3-5 weeks on Lind III medium and from 3-6 weeks on P medium, where autolysis is not evident. It would appear, therefore, that at least part of the tuberculoprotein, especially in the filtrates of the younger cultures, had been formed not because of autolysis of the cells, but by some other process.

In the present work, only the tuberculoprotein content of the culture filtrates has been considered. A subsequent paper will report the results of extraction experiments with the bacteria and measurement of the tuberculin activity of some of the culture filtrates and extracts.

#### SUMMARY

Glycerol broth and some synthetic media, including Sauton, Long and Dorset media, were inoculated with three virulent strains of *Mycobacterium tuberculosis*. After incubation at 38° C for one to nine weeks, the weight of culture, pH and protein content of the culture filtrate were determined for the heat-sterilized cultures.

*et al* 1958), but direct experimental proof of this has not been demonstrated and the actual mechanism of their formation remains obscure (Takehara 1957). Frequently culture filtrates with pH 4-5 have lower protein content than filtrates with pH 7-8 (Wong 1937, McIntosh & Konst 1943, 1949, Svenkerud 1955, Paterson *et al* 1958, Magnusson & Bentzon 1958, Asami *et al* 1959a). In contrast to observations by Szucs 1955, McIntosh & Konst 1949 and Svenkerud 1955 found no relationship between the total bacterial crop of different strains of *M. tuberculosis* and the yield of tuberculo-protein obtained on synthetic medium.

In the present experiment, the protein content of the culture filtrate increased regularly with the growth of the bacteria during three to four weeks. At that time the largest yields of tuberculo-protein were found in the glucose medium, probably due to the rapid growth of the tubercle bacilli in this medium. For older cultures, either Lind bII, Long or glucose medium showed the largest content of tuberculo-protein, dependent on the strain and age of culture. On Sauton medium the yield of tuberculo-protein was small, irrespective of age of culture.

Considering all the media as a whole, the total crops of bacteria were somewhat larger with U1921 than with E9656. However, the protein yield obtained with E9656 was on an average nearly twice as large as that obtained with U1921. For all three strains, about the same total crops of bacteria were obtained on Sauton, Long and glucose media, but the protein yield on Sauton medium was less than half of that in the two other media. Thus, in the present work, as in the reports of McIntosh & Konst 1949, and Svenkerud 1955, there is no relationship between the total bacterial crop of bacteria and the protein content of the culture filtrate.

The pH variations contribute to the varying protein content of some of the culture filtrates from the fully-grown cultures in the present experiment. This is evident in Fig. 1, which shows that the protein yield with strain T3487 on Sauton, Long, Lind bII and glucose media decreases and increases according to the changes in pH, with incubation periods longer than four to six weeks. The same correlation probably also exists when E9656 is cultured on Sauton medium (Fig. 3).

Furthermore, the small yields of tuberculo-protein with the fully-grown cultures of all three strains on Sauton medium are probably also due to the low terminal pH values. This has been shown more directly in subsequent experiments by varying the pH in fully-grown cultures of tubercle bacilli on Sauton medium immediately prior to sterilization, and by measuring the yields of tuberculo-protein obtained in the culture filtrates.

However, as regards strain U1921 cultured on Long and Lind bII media (Fig. 2), the correlation between pH and the protein content of the culture filtrates is not evident.

Magnusson & Bentzon (1958) found a definite relationship between pH and tuberculin yield for strain E9656 and less dependence for

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The crops of bacteria obtained were larger on the synthetic media than on glycerol broth. Within the synthetic media group, differences were found both in total bacterial crop and in growth rate of the bacteria, the largest crops being obtained in media with a high content of asparagine and potassium dihydrogen phosphate (Lind bII and P medium).

As regards the yield of tuberculo-protein in the culture filtrates, none of the synthetic media was consistently better than the others, the results of the comparison being dependent on the strain and the age of the culture. However, the yield of tuberculo-protein on Sauton medium was always the lowest.

No simple relationship was found in the fully grown cultures between the total crop of bacteria and the protein content of the culture filtrates. The protein yield for one of the strains could be twice as large as that of another, although the total bacterial crop was not larger.

The culture filtrates with pH 5.6 had a lower protein content than those with a higher final pH, and the low protein yield on Sauton medium was due to the low final pH in this medium.

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IV 7 ml sube 1 ml id and 2 ml iv given on the same day no booster  
 The animals in all 8 groups were bled by heart puncture 1 week after the last injection. The sera were inactivated at 56° C for 30 min and stored at -20° C

### Incubation Temperature

A temperature of 34° C was used for growth and incubation of the LSc 2ab strain while 37° C was employed for the Mahoney strain

### Serum Titrations

0.25 ml of 2 fold serum dilutions were mixed with 0.25 ml virus dilution containing 160-200 TCID<sub>50</sub> per 0.2 ml. After incubation for one hour 0.2 ml amounts of each mixture were inoculated into two MK roller tubes. The tubes were rotated for at least 4 days and were read on the 4 and 7 day. The serum titres were calculated from the 7 day's reading using Harber's method

### Plaque Technique

Monolayers of Mk cells were grown in Brockway bottles. These bottles have a flat side with an area of 320 sq cm.

The bottles were inoculated with  $2.3 \times 10^6$  versenated cells in 20 ml of Hanks' BSS with 0.5 per cent lactalbumine hydrolysate and 2 per cent calf's serum. The cell layers were used for inoculation when confluent i.e. on the 3 or 4 day.

After washing with Earle's BSS the cell layer were inoculated with 0.2 ml of virus dilution containing  $10^2$ - $10^3$  PFU (plaque forming units) and were subsequently incubated for 1 hr at 34° or 37° C.

The agar overlay then added consisted of

A 2.2 per cent Noble's agar melted and kept at 43° C

B  $2 \times$  Earle's BSS containing 1 per cent lactalbumine hydrolysate 0.2 per cent yeast extract 0.2 per cent bovine albumin and 0.005 per cent neutral red pre heated to 37° C in a water bath

A and B were mixed in equal portions and poured into the bottles 10 ml per bottle the thickness of the overlay being 3 mm. The composition of the overlay is given in Table 2.

Plaque counts and mean diameters were read on each of the subsequent 7 days.

## RESULTS

The antisera were titrated simultaneously in one experiment. The logarithms on the base of 2 of these titres are recorded in Table 1. Some general trends may be seen from this table.

- 1) The serum titres are found to increase with the length of the immunization period but they are not correlated to the number of injections nor to the total amount of virus employed in the present experiments i.e. 4 ml containing  $10^{6.9}$  TCID<sub>50</sub> per 0.2 ml ( $= 10^{8.2}$  TCID<sub>50</sub> LSc,2ab virus when using method II) and 10 ml containing  $10^4$  TCID<sub>50</sub> per 0.2 ml ( $= 10^{9.1}$  TCID<sub>50</sub> Mahoney virus when using method I and IV).

## SERODIFFERENTIATION BETWEEN TYPE 1 POLIOVIRUSES WITH ANTISERA PRODUCED IN RABBITS USING FOUR DIFFERENT IMMUNIZATION SCHEMES

By

KNUD SIBONI and HERDIS VON MAGNUS

Received 23 II 63

Work on the epidemiology of poliomyelitis and especially the need for differentiation between wild and attenuated vaccine strains has brought forward various methods for intratypic serological differentiation. McBride (4) used neutralization kinetics based on the work by Dulbecco *et al.* (1). Wecker (7) and Nakano & Gelfand (5) employed plaque neutralization, and Wenner (8) and Gard (2) found end-points from roller tube neutralization tests usable.

The present work was done with the purpose of finding a practical and preferably quick method for production of rabbit antisera, which would prove useful for serodifferentiation of various type 1 poliovirus strains.

### MATERIALS AND METHODS

#### Strains of Virus

- a) Mahoney V-1871 2 monkey kidney (MK) tissue culture passage
  - b) J Sc 2ab V-4116 1<sup>st</sup> MK tissue culture passage
- Both strains were kindly supplied by Dr. Albert B. Sabin.

#### Stock Viruses

MK tissue cultures in Roux bottles were grown in Hanks BSS (basic salt solution) with 0.5 per cent lactalbumine hydrolysate and 2 per cent calf's serum. They were changed into medium 199 before use. After complete degeneration of the cells the tissue culture fluid was harvested and frozen at -20° C in 1 ml portions. The two suspensions were labelled S 9 Mahoney and S 10 J Sc 2ab. They had titres of  $10^{7.4}$  TCID<sub>50</sub> per 0.2 ml and  $10^{6.9}$  TCID<sub>50</sub> per 0.2 ml respectively.

#### Antisera

Groups of 11 rabbits weighing 3000-3500 g were immunized with one of the two type 1 viruses in 4 different ways:

- I 9 ml subcutaneously (subc) and 1 ml intradermally (id) and 12 weeks later 1 ml intravenously (iv). This method has been used by Sabin (6).
- II 1 ml iv weekly for 3 weeks and 4 weeks later 1 ml iv.
- III 0.5 ml iv every 2 days during 2 weeks and 1 week later 1 ml intraperitoneally. This method has been used by McBride (4).

- b) Sera from rabbits immunized with the LSc,2ab strain show a higher titre against the homologous strain as compared to the heterologous (Mahoney) strain. However, the Mahoney strain is neutralized by LSc sera nearly to the same extent as by Mahoney antisera.

Sera from rabbits immunized with the Mahoney strain have about the same titre against the homologous and heterologous virus strain. From this rule Mahoney antisera 13 and 102 are striking exceptions as they show lower titres against the heterologous strain.

- c) Sera from rabbits immunized only once (method IV) sometimes have ill-defined end points, and single tissue culture tubes may show viral cytopathogenic effect at a much higher serum concentration.

To make sure that this degeneration was not due to viraemia in the rabbits at the time of heart puncture, four of these sera were diluted in saline and inoculated into roller tubes. No cytopathogenic effect appeared in these control tubes.

### *Estimation of Serological Difference*

In calculation of serological differences it is practical to use the following formula as employed for instance by Wenner (8)

$$\frac{\text{titre of serum 1 against virus 1}}{\text{titre of serum 1 against virus 2}} \times \frac{\text{titre of serum 2 against virus 2}}{\text{titre of serum 2 against virus 1}}$$

For identical strains this product is 1, for antigenic different strains it is  $>1$ . The calculations are facilitated when the above formula is transformed to logarithms on the base of 2

$$D = \log_2 \frac{\text{titre of serum 1 against virus 1}}{\text{titre of serum 1 against virus 2}} + \log_2 \frac{\text{titre of serum 2 against virus 2}}{\text{titre of serum 2 against virus 1}} > 0$$

This procedure of calculating is reasonable as the

$$\log_2 \frac{\text{homologous titre}}{\text{heterologous titre}}$$

is distributed normally ( $\chi = 1.85, f = 5, 0.80 < P < 0.90$ )

Accordingly, in the text below the figures recorded are not the actual titres but their logarithms on the base of 2. D is a measure for antigenic difference.

TABLE 1

*Serum Neutralization Titres Obtained in Groups of 6 Rabbits Immunized in 4 Different Ways with Mahoney or LSc 2ab Virus The Table Shows the Individual Titres Recorded as Logarithms on the Base of 2*

The  $\frac{\text{homologous titre}}{\text{heterologous titre}}$  is a measure for antibody specificity

Method of immunization	Rabbit number	Virus		Homologous titre
		Mahoney 125 TCID <sub>50</sub>	LSc 2ab 100 TCID <sub>50</sub>	Heterologous titre
I (12 weeks) Mahoney	1	16	15	1
	2	15?	16	—1
	3	13.5	13.5	0
	4	15.5	16	—0.5
	5	>16	14.5	>1.5
	6	>16	>16	—
I (12 weeks) LSc 2ab	7	13.5	16	2.5
	8	13.5	15	1.5
	9	14	>16	>2
	10	>16	>16	—
	11	12.5	13.5	1
	12	12	15.5	3.5
II (8 weeks) Mahoney	13	12.5	<10	>2.5
	14	13.5	15	—1.5
	15	14	14	0
	16	11.5	12.5	—1
	17	13	13	0
	18	13.5	14	—0.5
II (8 weeks) LSc 2ab	19	11	14	3
	20	10.5	14.5	4
	21	14.5	16	1.5
	22	10	13	3
	23	13	13.5?	0.5
	24	13.5?	16	2.5
III (4 weeks) Mahoney	93	13	12.5	0.5
	94	10.5	11.5	—1
	95	11.5?	11	0.5
	96	11?	10	1
	107	12	12?	0
	108	9.5?	10.5	—1
III (4 weeks) LSc 2ab	85	10.5	11.5	1
	86	9	12	3
	87	11	13	2
	88	10.5	12	1.5
	89	10	14	4
	90	10	11.5	1.5
IV (1 week) Mahoney	97	11.5	10?	1.5
	98	9	15	0.5
	99	12.5	10.5	2
	100	9.5?	9?	0.5
	101	9.5?	10?	0.5
	102	13	8.5	4.5
IV (1 week) LSc 2ab	91	11.5?	13?	1.5
	92	8.5?	12.5	4
	103	8	13	5
	104	9?	11.5	2.5
	105	12?	13?	1
	106	10.5?	13?	2.5

As the neutralization titrations were repeated for the sera from rabbits immunized by method II and III  $s_y^2$  could be estimated

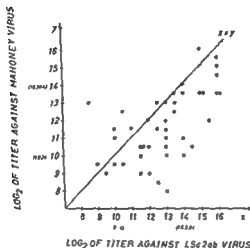
$$s_y^2 = 0.37$$

and consequently

$$s_y = 0.61 \text{ (log units)}$$

$$s_y^2 + s_L^2 = 1.91$$

CORRELATION BETWEEN SERUM TITERS AGAINST  
MAHONEY VIRUS AND LSc2ab VIRUS IN RABBITS IM-  
MUNIZED WITH MAHONEY VIRUS OR LSc2ab VIRUS



• RABBITS IMMUNIZED WITH MAHONEY VIRUS

○ RABBITS IMMUNIZED WITH LSc2ab VIRUS

Fig 1

### Plaque Neutralization Tests

It was found of practical importance to examine whether sera produced within one week could be used for differentiation in plaque neutralization tests. For these studies sera which had been found to have a high  $\frac{\text{homologous titre}}{\text{heterologous titre}}$  in roller tube neutralization tests were chosen.

The serum concentration required in the agar overlay to give a reasonably good effect was found to be between 10 and 80 units/ml, the variation supposedly being caused by the wide variation of serum titres ( $s_y^2 = 0.37$ , vide supra).

Table 3 illustrates an experiment of this kind. The figures give the

TABLE 2  
 $\log_2 \frac{\text{Homologous Titre}}{\text{Heterologous Titre}}$

Means,  $m$ , Standard Deviations,  $s$ , and Mean Errors,  $e$ , of the Figures Listed in the last Column of Table 1

Method of immunization		I 12 weeks	II 8 weeks	III 4 weeks	IV 1 week
Virus*					
Mahoney	$m$	0.20	-0.08	0.00	1.25
	$s$	1.04	1.39	0.83	1.89
	$f$	4	5	5	5
	$e$	0.58	0.70	0.42	0.95
LSc2ab					
LSc2ab	$m$	2.10	2.42	2.17	2.75
	$s$	0.97	1.26	1.12	1.51
	$f$	4	5	5	5
	$e$	0.56	0.50	0.56	0.76
$D = m(\text{Mah}) + m(\text{LSc})$		2.30	2.34	2.17	4.00
$s$		1.42	1.86	1.40	2.41
$e$		0.71	0.83	0.63	1.03
$f = \frac{D}{e}$		3.24	2.82	3.53	3.70
Number of rabbits necessary to demonstrate antigenic difference, $D > 0$ ( $P < 0.05$ )					
		$2 \times 2$	$3 \times 2$	$2 \times 2$	$2 \times 2$

The means,  $m$ , standard deviations,  $s$ , and mean errors,  $e$ , of

$$\log_2 \frac{\text{homologous titre}}{\text{heterologous titre}}$$

are listed in Table 2. It will be seen, that for all 4 methods of immunization the sum of the two means =  $D$  differs significantly from 0. Furthermore, the results show that this antigenic difference would be demonstrable using only 2 or 3 rabbits per virus strain instead of the 6 employed for each group in the present experiments.

The standard deviations,  $s$ , of  $D$  only differ slightly for the methods of immunization (Bartlett's test  $\chi^2 = 1.65$ ,  $f = 3$ ,  $0.30 < P < 0.40$  (Hald (3))), and therefore, a mean  $s_D$  is calculated

$$s_{D'}^2 = 3.39 \quad \text{The mean of } D \text{ is } 2.70$$

$s_{D'}^2$  is the sum of 4 times the variance on a neutralization titration in roller tubes  $s_{\frac{1}{2}}^2$ , and the variances in antibody specificity in rabbits immunized with Mahoney virus  $s_{\frac{1}{4}}^2$  and with LSc2ab virus  $s_L^2$ ,

$$s_{D'}^2 = s_{\frac{1}{4}}^2 - s_1^2 + 4s_L^2 = 3.39$$

To obtain more complete information one has however to examine sera prepared from the unknown virus strain with sera against known virus strains in cross neutralization tests. It is therefore of practical value that the rabbit antisera which were collected one week after one solid virus dose appear to be as specific as the sera obtained after a longer immunization schedule and that the titres of such one week sera are satisfactory.

It has been a general experience (*McBride* (4) *Nakano & Gelfand* (5)) that Mahoney antisera neutralize LSc 2ab virus and Mahoney virus to the same extent. It was therefore surprising that two of the Mahoney antisera (Nos 13 and 102) from the present experiments were found to be very Mahoney specific. This specificity was not due to Mahoney antibodies in the two rabbits before immunization since a 1:100 dilution of serum collected before the immunization was not able to neutralize 25 TCID<sub>50</sub> Mahoney virus in roller tubes.

Specific sera of this kind are of course very precious for antigenic analysis as it is evident from Table 3. In the plaque neutralization experiment recorded here a good differentiation was obtained using only the number of plaques as did *Nakano & Gelfand* (5). Also the size of the plaques can be used and may give a sharper differentiation even if number and size seem to be rather closely correlated (*Wecker* (7)).

In a new series of experiments the 1 week immunization technique was used (unpublished data). In these experiments the antigenic difference between the Mahoney and LSc 2ab viruses was demonstrable in roller tube neutralization tests as clearly as in the experiments recorded here ( $D = 2.33$ ,  $s^2_D = 1.475$ ,  $t = 4.66$ ) but none of the six new Mahoney antisera were as Mahoney specific as sera 13 and 102.

Antigenic analysis of wild poliovirus strains using the 1 week immunization method for immunization of rabbits is in progress.

#### SUMMARY

Groups of 10 rabbits were immunized with the Mahoney and LSc 2ab polioviruses using 4 different dosage schemes. The antibody titres were found to increase with the length of the immunization period. However the four methods of immunization resulted in sera equally suitable for distinguishing one virus strain from the other in cross neutralization tests in roller tubes. For epidemiological use it is therefore suggested to employ sera produced in one week after the injection of  $10^4$ – $10^5$  TCID<sub>50</sub>. In plaque neutralization tests such 1 week sera gave a satisfactory degree of differentiation.

Mahoney antisera are usually observed to neutralize Mahoney virus and LSc 2ab virus to the same extent but among the 24 Mahoney sera produced in the present experiments two contained Mahoney specific antibodies. Pre-immunization sera from these two rabbits contained no antibodies against Mahoney virus.



TABLE 3

*Plaque Neutralization Tests (37°) Using 1 Week Sera Against Mahoney Virus LSc,2ab Virus and a Strain 10077IV from a Vaccinated Person*

Experiment read day	Virus					
	Mahoney		LSc 2ab		100-IV	
	2	3	2	3	2	3
Virus control	13×10	18×10	12×10	18×10	30 III	4×10

## Sera

## Mahoney

## Rabbit 102

40 AU

20 AU

0

9

22

58

74

137

30

44

49

86

80

114

## LSc,2ab

## Rabbit 103

40 AU

20 AU

40

65

0

2

10

19

55

117

12

23

32

86

number of plaques on the last day when Mahoney plaques had not yet appeared in the bottle with Mahoney serum, and also the readings from the following day. Incorporated in the experiment was a type 1 poliovirus strain, 10077IV, isolated from a healthy child 3 weeks after oral vaccination with LSc,2ab. It may be seen that bottles with 40 AU per 0.2 ml agar overlay demonstrated the antigenic difference. If 20 AU per 0.2 ml had been the only concentration used, the difference might not have been demonstrable. The faecal strain appears to be antigenically different from the Mahoney strain but similar to the LSc,2ab, as it is suppressed to about the same degree as the LSc,2ab strain both by Mahoney antiserum 102 and by LSc,2ab antiserum 103.

In plaque tests not included in the present report the six 1-week Mahoney sera inhibited Mahoney virus at least to the same extent as LSc,2ab virus. Of the six 1-week LSc,2ab sera, four (Nos 92 103 104-105) inhibited LSc,2ab virus at a dilution not inhibiting Mahoney virus, but two (Nos 91-106) gave no differentiation.

## DISCUSSION

The antigenic specificity of poliovirus subtypes is a useful and rather stable marker. The differentiation may be carried out using known specific antisera, the degree of neutralization of known virus strains being compared to the neutralization of the virus isolated from a patient.

To obtain more complete information one has however to examine sera prepared from the unknown virus strain with sera against known virus strains in cross neutralization tests. It is therefore of practical value that the rabbit antisera which were collected one week after one solid virus dose appear to be as specific as the sera obtained after a longer immunization schedule and that the titres of such one week-sera are satisfactory.

It has been a general experience (McBride (4) Nakano & Gelfand (5)) that Mahoney antisera neutralize LSc 2ab virus and Mahoney virus to the same extent. It was therefore surprising that two of the Mahoney antisera (Nos. 13 and 102) from the present experiments were found to be very Mahoney specific. This specificity was not due to Mahoney antibodies in the two rabbits before immunization since a 1:2 dilution of serum collected before the immunization was not able to neutralize 25 TCID<sub>50</sub> Mahoney virus in roller tubes.

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# TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting December 1-2, 1961

Received 09 11 63

## 1 M Vastell THE GENERAL APPEARANCE OF THE BRONCHIAL MUCOSA IN BRONCHIAL CANCER

The bronchial mucosa has been investigated in 50 cases of bronchial carcinoma and 56 control cases. Basal cell hyperplasia was often found in both series. A similar alteration (pretransitional epithelium) and also "slit formation and expulsion" were however more common in the cancer material. A slit is formed underneath the surface cells resulting in a low epithelium without cilia. This probably provides the histopathological explanation for Papanicolaous Ciliocytophthoria. Keratinized squamous epithelium was found in carcinoma cases only and always within the tumour. Papilloma like stromaproliferations ("micropapilloma") had occurred in 60 and 100 per cent respectively of the cases while metaplastic epithelium (formed via basal cell hyperplasia or low epithelium) was found in 80 and 34 per cent respectively. Advanced epithelial atypia and extensive metaplasia was observed only in the cancer series and the former only in connection with transitional metaplasia. This suggested the existence of a generalized epithelial disturbance in the cancer cases.

## 2 Th Berge and O Grönroft CYTOLOGICAL DIAGNOSIS OF PLEURAL MESOTHELIOMA

## 3 O Grönroft HISTOPATHOLOGICAL DIAGNOSIS OF NEEDLE BIOPSIES FROM THE PROSTATE GLAND (prel communication)

## 4 A Bäckgren, L E Johansson, A Nordén, S Rubarth and E Winqvist SPONTANEOUS LEUCOSIS IN DOGS

In 5

" 5 10 years of age. The mean age was 6.7 years. The mean age for the whole autopsy material during this period was 4.32 years. Sex distribution of the whole material was 51.5 per cent males and 48.5 per cent females. In the leucosis material 58.8 per cent were males and 41.2 per cent females. The disease seems to be predominant in males, this is however not significant. As carcinoma in dogs leucosis appears most frequently in boxers and in Airedale terriers. In connection with the advanced mean age this perhaps may be an expression for the blastomatous character of leucosis.

## 5 *B. Isaerlöff* TRANSPLANTATION OF CHROMOSOME LABELED LEUKAEMIA CELLS

A transplantation form of virus induced erythroleukaemia in the fowl can be obtained by inoculating the chick with intact leukaemia cells. The host's own bone marrow cells which are the target for the virus, may, however, be infected by the virus released from the leukaemia cells and thereby undergo leukaemic transformation. In its final stage the disease thus developed would then be a combination of transplantation and virus induced leukaemia.

The occurrence of transplanted and autochthonous leukaemia cells in the body has been studied by chromosome analysis. Hence the cells of which have 11 macrochromosomes and an unpaired fifth chromosome were inoculated with leukaemia cells of cocks which have 12 macrochromosomes and paired fifth chromosomes. Three and four days after such inoculation the animals showed varying degrees of transplanted leukaemic proliferation in the bone marrow, and in the spleens the infiltration was in most cases due entirely to the proliferation of the transplanted cells. On the fifth day a few of the host's own cells were also recognized in the spleen and the bone marrow contained a mixture of autochthonous and transplanted leukaemic proliferation. It was concluded that during the first days the animals can develop a fairly pure transplantation leukaemia on which a virus induced component is subsequently superimposed.

## 6 *G. Hultquist and J. Thorell* TRIALS WITH PANCREAS TRANSPLANTATION IN RATS AND GUINEA PIGS

Pancreas tissue from rats or guinea pigs with pancreatic duct ligation and containing islet tissue but no other remains of exocrine parenchyma except ducts was implanted in the anterior chamber of the eye. The transplantations were carried out both in the animals with duct ligation and in other members of the same litter. After one month islet tissue showing signs of growth was found in many of the rats, about 90 per cent of the case of autologous transplantation and about 30 per cent of the others. Much poorer results were shown by the guinea pigs in which the survival time was 9-10 days at the maximum.

The grafts always contained  $\beta$  cells showing a pseudo-iodine reaction but  $\alpha$  cells were present only in some cases. In a few  $\alpha$  cells a positive reaction was obtained with Voigt's sulfide silver method.

Karyometry showed nuclear enlargement in both  $\beta$  and  $\alpha$  cells with maximum enlargement in 3-4 day grafts. The change gradually decreased and after 3-4 weeks the values corresponded to those of the normal pancreas.

## 7 *G. Nathorst Windahl* DIABETES IN PANCREATICTOMIZED RABBITS

Subtotal pancreatectomy in rabbits produces a relatively mild type of diabetes with glycosuria, polyuria, hyperglycaemia and an initial loss of weight. There is often an overproduction of ketone bodies but severe acidosis or coma does not develop. The animals are able to live for long periods without insulin and yet often maintain an almost constant rather low body weight in spite of pronounced glycosuria.

Total or approximately total pancreatectomy however produces a severe diabetes with massive ketonaemia and ketonuria. Metabolic acidosis and coma develop in a few days unless insulin is given.

These results are at variance with the opinion of Greeley as cited by Houssay

(Comparative Endocrinology, Wiley, New York 1959) who stated that total pancreatectomy produces a mild type of diabetes in rabbits

The pancreatic rests in the subtotally depancreatized diabetic rabbits have been studied. Pictures suggestive but not convincing of so called acino insular transformation have been obtained. Glycogen infiltration in hydropic  $\beta$  cells has been shown

8 S Folkmer and F Annlzon IS COBALT CONCENTRATED IN PANCREATIC ISLET TISSUE?

9 J Zamboni ELECTRON MICROSCOPICAL INVESTIGATION ON THE EARLY STAGES OF POLYOMA VIRUS INFECTION IN MICE

A pellet obtained by ultracentrifugation of medium from polyoma infected tissue culture and very rich in virus particles mostly attached to fragments of tissue culture cells was resuspended in a small quantity of fluid and subcutaneously injected in adult mice

15 minutes 45 minutes and 24 hours later subcutaneous tissue was excised from the injection area and studied with the Electron Microscope 15 minutes after the injection virus particles were observed in the cytoplasm of the tissue cells. They had been transported through the cell membrane as single units by pinocytotic activity or in clusters attached to the injected cell fragments by phagocytosis

After 45 minutes few particles were detectable intranuclearly whereas 24 hours later the nuclei of many cells were literally loaded with virus. The degeneration of the infected cells leads to a fragmentation of the elements their debris rich in virus particles are phagocytosed by healthy cells with consequential spreading of the infection

10 A Hergatz and C G Bergstrand A Engstrom and A M Herrlin RENAL CHANGES DURING TRIDIONE TREATMENT

A nephrotic syndrome is a rare complication to treatment of epilepsy in children with Tridione  $\beta$ . Experimental investigations also indicate that Tridione in large doses may have a nephrotoxic effect. In order to find out if slight and clinically non observed damage to the kidneys is a frequent complication we have investigated seven cases aged 15-17 years which have been treated with Tridione for periods between three months and eight years. Continuous renal examination showed only slight and transient haematuria or proteinuria in three of the cases

Light microscopy of renal needle biopsy material showed no glomerular or tubular lesions

Electron microscopy of the glomeruli showed wide variations in the appearance of the capillary epithelial cells. These are regarded as physiological variations and not as pathological phenomena

The investigation has not shown any signs of renal damage which could be assigned to the treatment

11 B Thorell LOCALIZATION AND DETERMINATION OF RESPIRATORY ENZYMES IN THE SINGLE LIVING CELL

With regard to the fundamental energy linked cell reactions, e.g. the cell respiration, our knowledge about the functions of the various cell structures has

so far mainly been based on bulk analyses of isolated tissue fractions. The present paper describes some recent developments of the microspectrographic technique which permit quantitative *in vivo* assay of respiratory enzymes in localized areas of  $15 \mu$  in diameter in the single intact cell. The detectability is equivalent to 6000 molecules of cytochrome *b*. Soret spectra of both oxidized and reduced cytochromes can be demonstrated depending on whether the cell is in an aerobic or an anaerobic state. Measurements on the grasshopper spermatocyte and on isolated liver and kidney cells have given information about the metabolic state of the respiratory system even of the single structures within the living cell.

12 A. F. Åström EXPERIMENTS ON ALLERGIC ENCEPHALOMYELITIS

13 S. Sjögr. ASEPTIC MENINGOENCEPHALITIS AND MYOCARDITIS (PROBABLY LOCKSACKIE) IN A NEW BORN CHILD

14 S. Lundberg and O. Grøntoft A CASE OF BILATERAL SYMMETRICAL CALCIFICATIONS OF THE BRAIN

15 L. Bjersing and A. E. Borglin EOSINOPHILIA IN UTERUS

In recent years the authors have occasionally seen massive collections of eosinophilic leucocytes in the human uterine myometrium. Fifteen cases were studied in detail. Two groups were used for comparison. In one of the groups amputation of the uterus or cervix had been preceded by curettage or biopsy but not in the other. Eosinophilic granulocytes were observed—also in the controls—usually around vessels or between muscle bundles in the myometrium and showed no correlation with the amount or site of other inflammatory cells. No explanation can be offered for this abnormal eosinophilia which has as far as we know not been described before. The eosinophilia was slight and it was rare when the interval between the interventions was more than 3 weeks. Animal experiments in progress have shown absence of eosinophilic leucocytes in the uterus of spayed rats and eosinophilia in spayed animals treated with oestrogen, the degree of eosinophilia varying closely with the dose given.

16 G. Fickera and I. Hagerstrand LYMPHATIC CHANGES IN CHRONIC PULMONARY CONGESTION

A post mortem series of chronic pulmonary congestion (100 cases of mitral stenosis, 75 cases of pulmonary congestion because of hypertension or myocardial lesions) was studied. Compared with the controls (healthy lungs from 50 cases of different ages) fibrous mural thickening of the lymphatics was found at different levels (perivascular, peribronchial, interlobular and intrapleural) in 70 per cent of the cases of pulmonary congestion. This lymphatic fibrosis appeared to develop together with other pathological signs of chronic congestion and was probably not a primary lesion. The fibrosis was probably due to increased drainage of fluid.

17 I. Hagerstrand and F. Linell SARCOIDOSIS AND POLLEN

Experiments were performed to check the hypothesis put forward in recent years that sarcoidosis might be due to fir tree pollen. All of the pollen studied (different sorts of fir, tree, pine, beech, oak, maple, alder, *Isopodium timothy grass*) proved

acid fast Pollen was suspended in physiological saline and injected subcutaneously and intraperitoneally into rabbits (33 animals) The animals were killed 18 months later Foreign body granulomas developed around the injections but no epithelioid cell granulomatosis was observed The experiments provided no support to the hypothesis of a causal relationship between pollen and sarcoidosis

18 *L H Larsson* ENDOCARDIAL MYXOMA WITH MALIGNANT DEGENERATION

A woman without known rheumatic fever had for 3 years had progressive cardiac failure clinically interpreted as combined mitral and aortic disease During the last few months widespread skeletal lesions of malignant character were observed Necropsy revealed a myxomatous tumour at the fossa ovalis in the left atrium The endocardium in the atrium was thickened the mitral orifice was stenosed the mitral valves and chordae tendinae were rigid and thick and the aortic valves showed diffuse fibrous thickening The left lung showed a hilar tumorous nodule with peripheral extensions along the vessels Numerous metastases were seen in the heart wall in the skeleton and in the left ovary The atrial tumour exhibited the histological characteristics of an endocardial myxoma with sarcomatous degeneration The rest of the atrial wall the mitral and aortic valves chordae tendinae and the pulmonary veins showed marked fibrous thickening with a surface layer of anaplastic tumour cells The relations between the tumour and the valvular and pulmonary lesions are discussed

19 *L Olding* PERINATAL BACTERIAL INFECTIONS IN AN AUTOPSY MATERIAL

20 *C Lundmark* GRANULAR ATROPHY OF THE BRAIN—OBLITERATING ARTERIAL DISEASE

21 *C Lundmark* CARCINOID TUMOUR WITH METASTASES IN THE HEART AND AORTIC STENOSIS

22 *B Larsson* A CASE OF POSTANGIOGRAPHIC Th DEPOSITION AND PRIMARY SQUAMOUS CELL CARCINOMA OF THE LIVER

A bilateral cerebral angiography had been performed with a total of 17 ml of Thorotrast Twenty seven years later the 53 year old male succumbed to a large necrotizing liver tumour squamous cell in type Autops total body scintillography had shown no uptake in the region of the spleen before a surgical splenectomy Metastases were found in regional lymph nodes the peritoneum the adrenal regions and in one thoracic vertebra There were no thorium dioxide deposits at the sites of injection The liver weighed 1800 g and revealed a moderate and mainly post necrotic scarring Thorotrast deposition was demonstrated in liver and spleen with routine methods and autoradiography The bone marrow was in a reactive state A moderate anaemia was present There were no oesophageal varices



# TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

*Meeting April 7, 1962*

## 1 *L-B Schnürer and S Stattin* THE VASCULAR SUPPLY OF TENTORIAL MENINGIOMAS

In some papers dealing with tentorial meningiomas vessels have been described seemingly arising from the carotid siphon. This prompted an investigation on the topography and distribution of these vessels, performed on autopsy specimens with the aid of a dissection microscope. The vessels were successfully injected and dissected in about 15 of the specimens. The number and course of the major branches were found to be rather constant. Thus there are two main branches, one arising from the infero-lateral aspect of the so called horizontal portion of the siphon, the other from the upper posterior aspect of the convexity of the proximal portion of the siphon. In most cases both main stems give off tentorial branches, in some only one of them does. The tentorial branch of the lateral main stem in most cases runs directly upward, enters the tentorium and continues backward near the free margin, sometimes up to the summit. The tentorial branch of the dorsal main stem is usually smaller and runs backward in the tentorium near its attachment to the petrous bone. These tentorial vessels are most often but not always too small to be visualized at angiography, but they may hypertrophy when contributing to the vascular supply of meningiomas and vascular malformations and thus be of angiographic and neurosurgical importance.

## 2 *C Åhrén* EFFECT OF DIENCEPHALIC LESIONS ON TESTIS IN ADULT RABBITS

Diencephalic lesions were produced in 248 adult male rabbits with the aid of Ranström's X-ray stereotaxic instrument in medial parts of the diencephalon from the anterior medial preoptic area to the rostral border of the mesencephalon. The animals were allowed to survive for 6 weeks, half of these were subjected to repeated subcutaneous injections of formalin during the final 3 weeks. On the basis of the size, localization and symmetry of the lesions determined histologically from serial sections of the diencephalon 140 animals were segregated into 10 groups. (Each about equally divided as to formalin treatment). The median eminence and pituitary gland were histologically intact in all animals.

*Results.* Some animals showed gross testicular atrophy not correlated to any particular lesion. Severe atrophy was only observed with formalin treatment probably a non specific stress reaction. There were no significant differences in the mean testicular weight in the 10 groups as compared with the control animals. Histological signs of altered spermatogenesis in some animals suggest disturbances in secretion of IHH. Such changes were most frequently observed in animals with

posterior diencephalic lesions. However, these results are preliminary and must be confirmed by further studies.

The results support the view that there is no localized "sexual center" in the adult male rabbit. However, posterior diencephalic lesions seem to induce alterations in spermatogenesis.

2 *P. Sourander* MORBUS KRABBE

4 *I. Hagerstrand* THALIDOMID INDUCED MALFORMATIONS

5 *H. Sunzel and L. Zettergren* LESIONS IN THE LIVER AFTER SURGICAL OPERATIONS IN THE UPPER PART OF THE ABDOMEN

6 *L. Enerbäck* HISTOCHEMICAL REACTIONS IN CARCINOMAS

7 *F. Arnason* AUTORADIOGRAPHY OF PITUITARY CELLS IN ADRENAL-ECTOMIZED WHITE RATS AFTER THE INJECTION OF ADENINE- $^{14}\text{C}$

Five male white rats weighing 150-200 grams were adrenalectomized. Observation times were 1, 2, 4, 8 and 12 days. Two hours before the end of each observation time the animals were injected with 0.05 mC adenine- $^{14}\text{C}$ .

The pituitaries were fixed in neutral formalin, embedded in paraffin and sectioned serially. Autoradiography was performed with strip film technique and Kodak AR 10 emulsion. The unstained autoradiographic specimen was mounted in a glycerol medium and examined under a photo microscope with a micron graduated mechanical stage. Labelled cells were photographed in transmitted light and in phase contrast. The film was removed and the section stained with Trichrome PAS technique. With the aid of the graduated stage and the previously taken photographs the same labelled cells were localized in the stained section.

About 70 labelled cells within random fields in all pituitaries had been so localized. One day after adrenalectomy 70 per cent of labelled cells were small chromophobes. With increasing time after adrenalectomy their percentage gradually diminished whereas the number of labelled large vacuolized chromophobes and amphophiles increased, reaching almost 50 per cent 8 and 12 days after adrenalectomy. Very few well granulated basophiles were labelled. The results point at the chromophobe cell as the site of new production of ACTH and it was suggested that these chromophobes gradually turned into vacuolized forms and so called amphophiles.

8 *P. Lunin* THE CARCINOGENIC ACTION OF COMPLEX IRON PREPARATIONS

9 *H. Röckert and L. Zettergren* LESIONS CAUSED BY BARIUM CONTRAST MEDIA

10 *I. Angerhäll and S. F. Fagerberg* SKIN BIOPSY IN DIABETES

Since the beginning of 1961 punch biopsies from the skin on the dorsal part of the basal phalanx of the 4th toe of diabetics have been examined. All specimens have been fixed in 10 per cent neutral formalin solution, about half of them being

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fixed also in 4 per cent basic lead acetate solution. The skin specimens were cut into 4  $\mu$  serial sections consecutive sections being stained with haematoxylin eosine van Gieson haematoxylin Hansen and MacManus periodic acid Schiff. So far we have examined biopsy specimens from 50 male and female diabetics of varying ages and from 35 non diabetics.

All but one of the diabetics exhibited dermal microangiopathy in the form of wall thickening and reduction in the lumen due to deposition of a strongly PAS positive substance which in van Gieson stained sections was a homogeneous mildly picrinophilic material. Some vessels displayed hypertrophic hyperchromatic nuclei between PAS positive fibrils of the basement membrane. The diabetic microangiopathy is usually most pronounced in the capillaries surrounding the sweat glands and in the papillae. The larger the vessels the more difficult it is to find characteristic lesions.

All but one of the controls showed in general a suggested or slight PAS positive reaction.

The facts that diabetic microangiopathy has now been encountered in the skin constitutes additional evidence that it occurs throughout the organism.

# TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

*Meeting December 1, 1962*

## 1 L Santesson ON THE CLASSIFICATION OF OVARIAN TUMOURS

At an international conference in Stockholm the following proposal was made  
The Conference recommends that the common epithelial tumours of the ovary be classified in the following manner

### 1 Serous cystomas

- a) Serous papillary cystadenoma benign (Figs 69 and 71)
- b) Proliferating serous papillary cystadenoma without stromal invasion (possibly malignant) (Figs 72 and 75)
- c) Serous cystadenocarcinoma all grades (Figs 76 and 81)

### 2 Mucinous cystomas

- a) Mucinous cystadenoma benign (Figs 87 and 90)
- b) Proliferating mucinous cystadenoma without stromal invasion (possibly malignant) (Figs 91 and 92)
- c) Mucinous cystadenocarcinoma all grades (Figs 93 and 95)

### 3 Endometrioid tumours

- a) 1
- b) Proliferating endometrioid adenoma and cystadenoma (possibly malignant) (Fig 102)
- c) Endometrioid adenocarcinoma all grades (Figs 77 and 105)

### 4 Undifferentiated carcinoma (cell type unknown)

[The numbers in parenthesis refer to figures published in Tumors of the Ovary and Fallopian Tube by Hertig A T & Gore H (Atlas of Tumor Pathology Section IX Fascicle 33 Tumors of the Female Sex Organs Part 3 Armed Forces Institute of Pathology Washington 1961)]

Usually in any given tumour only one type of epithelial cell is dominating. Occasionally when there is a mixture of different cell types the predominant type should determine the diagnosis

## 2 S Falkmer F Knutson and G E Toigt FURTHER STUDIES ON THE COBALT CONCENTRATING ABILITY OF PANCREATIC ISLET TISSUE

The recent discovery by quantitative scintillation determinations using  $^{54}\text{Co}$  that isolated islet tissue has approximately the same high ability as the liver to con

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1 The existence of a blank space after 3 a) is due to the fact that there is as yet no agreement as to whether some cases of ovarian endometriosis may properly be classed as true tumours or all must be regarded as simply displaced or ectopic endometrium

centrate cobalt (Veta Endocr 42 1967) prompted us to try further localization of the cobalt in the pancreatic islets. As conventional autoradiography had shown that the accumulated cobalt was easily dissolved from the tissue during fixation and embedding procedures we had to modify the histochemical and autoradiographical methods.

The isolated islet tissue of the marine teleost *Cottus scorpius* was cut in a microtome cryostat. Autoradiography was performed without the sections coming in contact with water. Other islets were gassed with hydrogen sulphide, sectioned in the microtome cryostat and subjected to a modified sulphide-silver procedure to detect precipitated cobaltous sulphide after extracting other heavy metal sulphides. The injected cobalt was localized to the islet parenchyma and not to the stroma. By comparing the autoradiograms and the sulphide-silver sections with adjacent sections stained with aldehyde fuchsin (for the visualisation of the B- and A cells) it was possible to show that the injected cobalt was confined only to the cells in the dark central region of the sculpin islets containing the B cells and the argyrophil A cells.

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The insulin antibody titre was assayed by measuring the capacity of serum to bind insulin- $I^{131}$ . After incubating the sera with insulin- $I^{131}$  for 5 days at 4° C the antibody-bound insulin was separated from the unbound insulin by paper electrophoresis according to Betson & Jalow. The antibody titres were assayed in blood obtained from the umbilical cord and from the mother on the same occasion.

In pregnant guinea pigs with relatively low insulin antibody titres the insulin binding capacity of the foetal blood was usually double that of the maternal blood at term.

The titres of three newborn children of insulin treated diabetic mothers were about equal to their mothers' titres.

This placental transfer of antibodies may be of some importance for the foetal insulin metabolism. Further studies on this subject are in progress.

### 5 P Sundelin and B Lagerlof DISEASES INDUCED BY MYELOID LEUKAEMIA VIRUS

Myeloid leukaemia virus can induce several different diseases in the fowl. The type of disease induced has been shown earlier to depend on the age of the bird and the amount of virus inoculated. An investigation into the pathogenic effect of the myeloid leukaemia virus revealed that our virus strain has a pathogenic spectrum similar to that described earlier and induces myeloid leukaemia, visceral lymphomatosis and kidney tumours. However, in our material an additional form of leukaemia, a subleukaemic anaemia, was observed. This form of the disease is characterized by lymphoid hyperplasia and occasional areas of necrosis in the bone marrow, a severe anaemia, a comparatively low number of leukaemic cells in the peripheral blood and siderosis of the spleen, indicating haemolysis.

An electrophoretic investigation of the plasma from leukaemic birds revealed that

chicks with a late onset of the disease dying at an age of more than one month had a significant increase of the gamma globulin fraction. This probably contains viral antibodies and may thus be responsible for the fact that the virus concentration of the plasma from leukaemic chicks decreases with increasing age of the birds. Young chicks with marked viraemia had normal amounts of gamma globulin. Chicks even young ones with the subleukaemic anaemia had high gamma globulin fractions which taken together with the lymphoid hyperplasia and the signs of haemolysis seems to indicate an immunologic cause of the disease.

#### 6 *T. Sallén* EXPERIMENTAL INVESTIGATION OF CANCER SPREAD TO THE LIVER IN RATS WITH LIVER CIRRHOSIS

Moderate liver cirrhosis induced by administration of carbon tetrachloride to rats increased the percentage of secondary intrahepatic growths following intraportal injection of tumour cells from Rous rat sarcoma. The number of tumour nodules in the liver was higher when the interval between the last injection of carbon tetrachloride and the injection of tumour cells was 4 or 6 weeks than when it was 2 weeks. A positive correlation was found between the extent of fibrosis in the liver and the number of tumour nodules.

After injection of tumour cells into a tail vein tumour nodules were seen in most cirrhotic but in no normal livers.

Liver cirrhosis had no effect on the growth of the tumour after direct intrahepatic injection of the tumour cells suggesting that the vascular changes in the liver are responsible for the increased number of intrahepatic secondary growths.

A possible increase in the number of tumour cells trapped in the cirrhotic livers can however hardly explain the higher percentage of takes in the liver. Another possibility is that the narrowing and obliteration of the smaller vessels prevented the tumour cells from coming into contact with Kupffer cells.

#### 7 *J. Zajicek* NEEDLE BIOPSIES AS A METHOD TO OBTAIN HUMAN TUMOUR MATERIAL FOR BIOCHEMICAL AND BIOLOGICAL INVESTIGATIONS

#### 8 *P. S. Persson* CYTOLOGIC DIAGNOSIS OF SUBACUTE AND CHRONIC THYROIDITIS

Thin needle punctures were carried out in 173 patients with goitres. The smears were stained according to the May Grunewald Giemsa method. Of 9 patients with the clinical picture of subacute thyroiditis 6 had the cytologic findings of subacute thyroiditis and 2 a suspected one. No definite diagnosis could be made in 1 patient. In 42 patients the cytologic diagnosis was chronic thyroiditis. In 24 patients the diagnosis was verified by histopathological examination, the rest had a typical clinical picture. There were no cytologic differences between various kinds of chronic thyroiditis.

The cytologic findings of subacute thyroiditis were:

- a. collections of polymorphonuclear leucocytes, histiocytes and mononuclear inflammatory cells
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The cytology of chronic thyroiditis is characterized by (1) proliferation and to a lesser degree (2) degeneration of the follicular epithelial cells (3) collections



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The cytologic findings of subacute thyroiditis vary with the phase of the disease and present the following features: (1) marked degeneration of the follicular epithelial cells, (2) proliferation of the follicular epithelial cells in the late phase of the disease, (3) foreign body giant cells, (4) collections of polymorphonuclear leucocytes, histiocytes and mononuclear inflammatory cells.

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11 *A Cederberg and G Jästberg* OXYGEN INTOXICATION

A girl of eighteen after some days of sore throat suddenly developed pain in the right side of the chest and heavy respiratory distress. On the third day she was taken to hospital where blood was found in the right pleura. She was given transfusions and treated with drainage of pleura later on tracheotomy and artificial respiration with increasing partial pressure of oxygen up to 52 per cent in the last hours. She died on the tenth day. Clinical diagnosis: Spontaneous haemo-pneumo-thorax.

At autopsy the lungs which weighed 830 and 870 grams were strikingly heavy and firm. No rupture or bleeding was found on the lung surface. Microscopically a dense fibrinous exudate filling the alveoli of right lower lobe was seen. Other parts showed widespread formation of hyaline membranes on alveolar septa.

Pulmonary hyaline membranes of adults are rare. They seem to occur without any certain relation to other diseases. The rôle of oxygen treatment has been discussed. Possibly hyaline membrane formation can be induced by thoracic surgery. Our case might have a similar mechanism with collapse of the right lung and compression of the left at spontaneous haemo-pneumothorax. Delay of treatment may have played a rôle.

12 *J Soderström* SPLENIC RUPTURE IN INFECTIOUS MONONUCLEOSIS

About 50 cases of splenic rupture in infectious mononucleosis are reported. The histological picture is described on the basis of a personal collection of 6 cases. Three of the 6 patients had died and 3 had been operated upon with success. The spleen showed intense proliferation of large mononuclear cells above all in the red marrow but also in the capsule and the trabecular network that had thereby become loose which probably facilitated the rupture. The cellular proliferation in the vessels was noteworthy. Large round cells were seen in the adventitia of the arteries and subintimally in the veins. Other organs showed similar changes. The picture was the same in all the cases studied and coincided with descriptions of earlier reported cases.

The most important disease to be considered in the differential diagnosis is leucosis. Careful pathological examination of the characteristic—though not pathognomonic—histological picture together with clinical and laboratory data enable a firm diagnosis of mononucleosis infectiosa.

13 *4 Roussing* GLIOMA INDUCED BY FOREIGN BODY

A 55 year old woman with bilateral symptoms of parkinsonism since 1951. 1956 pallidotomy right side leaving two spirals of tantal in the brain. 1959 left sided thalamotomy, no foreign bodies left behind. No relapse on left side, speedy recurrence on right side and progressing symptoms of increased intracranial pressure until death in 1962.

Autopsy: small-orange sized cyst in right sided field of operation. Two metal spirals were found in its wall. Walnut sized tumour in the right occipital lobe. Brownish discoloration in the left sided field of operation. Histology: tissue from right sided cyst wall and tumour and left sided lesion showing malignant glioma. No continuity proved between the different sites.

of lymphocytes lymphoblasts plasma cells reticulo endothelial stem cells and phagocytes Epithelioid cells may sometimes be seen

Difficulties in the differential diagnosis exist between thyroiditis and special types of thyrotoxicosis and malignant thyroid diseases

## 9 L G Wiman SULPHYDRYL CHEMISTRY AND FLUORESCENCE MICROSCOPY IN EXFOLIATIVE PULMONARY CYTOLOGY

Two new cytochemical staining procedures for exfoliative cytology are introduced With one method first published in 1959 by the author a high concentration of proteinbound sulphhydryl groups is demonstrated in exfoliated tumour cells In ordinary light microscopy these cells stain intensive blue and can be easily distinguished from surrounding normal cells which appear as pale shadows With a second method (1962) the ability of tetracycline to fluoresce in ultraviolet light is used to display tumour cells In a patient treated with tetracycline the substance is concentrated in tumours especially According to their conspicuous yellow green fluorescence exfoliated malignant cells can be easily identified in smears of sputum bronchial secretions aspirates from pulmonary tumours and pleural fluids If unfixed smears from patients not treated with tetracycline are immersed in a tetracycline solution a strong yellow fluorescence is induced in the tumour cells Upon the same material Bertalanffy's method was tried using fluorescence microscopy after staining with acridine orange Hereby certain malignant cells got a bright red fluorescence on account of a high content of RNA in cytoplasm and nucleolus which makes them easy to find during screening work However malignant cells exfoliated from a highly differentiated squamous cell carcinoma often have a low content of RNA For this reason they get a faint olive green or orange red fluorescence and may therefore be overlooked in the microscope This and some practical disadvantages gives the method a limited value in diagnostic cytology The results of all methods above has been correlated to and compared with the Papanicolaou staining procedure

## 10 A Gydell I Juhlin and J G Norden NOCARDIOSIS

A 41 year old female acquired an auto immunologic haemolytic anaemia and was treated with increasing doses of steroids (average dose during 1 year 29 mg of Deltacortril® per day) After 11 months she got fever and chest roentgen showed infiltrations which later cavitated No acid fast bacilli were found guinea pig inoculation was negative but nocardia was cultivated from bronchial secretion After heavy medication with penicillin the patient improved transiently but then she became worse with deterioration and epileptiform seizures Increasing sputum blocked the air passage On treatment with Elkosin® (sulphaisodimidin) the chest roentgen picture became almost normal and nocardia disappeared from the trachea But the blood values decreased and finally she became unconscious and died

*Autopsy* In the lungs solid yellow foci and a system of cavities were noted The kidneys showed several yellow streaks and a larger necrotic focus which continued into a perirenal abscess with greenish thick necrotic material Such abscesses were also found in all parts of the brain and in musculature (neck and leg) Nocardia could be demonstrated in cultures with material from brain and kidney but in no other organ

The changes of the oral mucosa were the same as in earlier reported cases. The polydysplastic case showed a peculiar discharge of well defined parts of connective tissue demarcated by ingrowing epithelial strands.

In the tooth germs heavy disturbances in the enamel formation were found such as metaplasia of the enamel epithelium with many epithelial whorls. In micro radiograms these appeared as globular irregularly mineralized structures. The enamel was considerably thinner than normal. In the case where the teeth had erupted they showed severe hypoplasias similar to those clinically seen in amelogenesis imperfecta. In all the cases the tooth germs showed the same disturbances. It is concluded that amelogenesis imperfecta may form a stigma in the reported forms of epidermolysis bullosa hereditaria.

#### 18 H Larsson ON MALIGNANT LIVER AND BILE DUCT TUMOURS IN CASES EXPOSED TO THOROTRAST AND AMONG WINEGROWERS WITH LONG STANDING EXPOSITION TO ARSENIC

The two groups were briefly presented as available in the literature. The non epithelial tumours were mainly discussed carcinomas being further referred to elsewhere. Some differences between the radioactive  $\text{ThO}_2$  and the chemically toxic  $\text{As}_2\text{O}_3$  were mentioned. The Th sarcomas appeared after an average time of 16 years and the As tumours after 20 years. The Tumour Development Time (TDT) in these human series represents Tumour Death Time in experimental series a Tumour Detectable Time might be registered.

It is obvious that different substances can induce similar tissue responses. In the present two sarcoma groups there is one cellular main response and one terminal lesion.

Human tumours possibly exogenously induced and certain characteristics in the response of some animal species to carcinogenic influence were considered to present a tentative basis for a further study of common denominators in the process of malignant development.

#### 19 S Dahlgren RENAL CELL CHANGES AFTER INFUSION OF LOW VISCOSITY DEXTRAN

During the last year a great number of cases with changes in the renal tubules of the type known as osmotic nephrosis have been found at post mortems at the Karolinska sjukhuset. 31 cases featuring such changes have been diagnosed and out of these 27 had been given so called low viscosity dextran (average molecular weight 40000) in amounts ranging from 100 to 4400 cc. The other four cases had been given rather large doses of various hexosis. It has not been possible to determine whether the tubular changes are accompanied by disturbances of function. In spite of the fact that the commonly used type of dextran (average molecular weight 75000) has been widely used only 23 cases with tubular changes have been reported in man after infusion of dextran of the latter type (Johnston & Lundy 1953, Vicery 1956 and Zettergren 1962). The great number of cases with histo-pathological changes in the renal tubules after infusion of low viscosity dextran seems striking.

#### 20 S Sjögt ISOLATED TOXOPLASMA MYOCARDITIS IN A CHILD

Autopsy report of a 5 year old boy who was found dead in his bed. The boy had often had infections of the respiratory tract. During the previous 6 months he had been fairly depressed.

implantation of tantal plates. It is suggested that neuroglia may give a malignant response to a similar stimulus.

#### 14 C Lundmark A FATAL CASE OF VARICELLA

#### 15 I Olding EPIDERMOLYSIS BULLOSA LETHALIS SOME MORPHOLOGICAL OBSERVATIONS

Sections of the skin from four infants who all died within nine weeks after birth and who had all been affected with epidermolysis bullosa lethalis were examined by conventional histological methods. Sections were stained with haematoxylin and eosin and Weigert's elastin stain. The argyrophilic reticulum fibers were visualized by Landau's method. Sections of skin from dead infants at different ages and without skin diseases were used as controls.

The early changes of epidermolysis bullosa lethalis were multiple intracellular vesicles in the basal layer of the epidermis. The subepidermal elastin plexus was practically absent but this was also the case in the controls. This coincides with the observations of Dick (J Anat 81 201 1947) who has shown that the subepidermal elastin plexus is practically absent in the normal skin in infancy. On the other hand the reticulum fibers in the uppermost dermis were well developed both in the cases of epidermolysis bullosa lethalis and in the controls. These reticulum fibers were prominent also at the margins of recent bullae where the basal layer showed a marked vesicular degeneration.

#### 16 B Robertson REPARATIVE PHENOMENA IN HYALINE MEMBRANE DYSPLASIA

In an autopsy material of 117 neonatal deaths varying in age between 6 hours and 6 days all displaying pulmonary hyaline membranes proliferative and reparative phenomena in the alveolar epithelium were observed in cases surviving more than two days. This agrees with the observations recently reported by Ross & Craig (Pediatrics 23 890 1962). Similar changes in the alveolar epithelium were also observed in four other autopsy cases, premature, varying in age between 10 and 24 days. The clinical picture in these four cases had been that of idiopathic respiratory distress since shortly after birth. Histological sections from the lungs displayed thickened alveolar walls containing fibrin blast like cells and excess of collagen and reticulin. No hyaline membranes of the usual type were observed in these sections although small amounts of hyaline material were present in some alveoli.

These findings reflect a process of repair which indicates previous damage to the alveolar walls possibly connected with the development of hyaline membranes. But since the author has also observed similar changes in the alveolar walls of 2-3 week old neonates without typical clinical history of idiopathic respiratory distress it is too early to draw any definite conclusions. A clinical analysis of these cases is in progress.

#### 17 T Arnulf & Bergenholt and O Olsson EPIDERMOLYSIS BULLOSA HEREDITARIA A HISTOPATHOLOGICAL STUDY OF TEETH AND ORAL MUCOSA IN THREE CASES

Demincralized sections and microradiographs in ground sections from the jaws of patients with polydysplastic and lethal forms of ebh were studied.

## OBSERVATIONS ON EFFECTS OF ILEUM RESECTION ON GRANULOCYTE COUNTS IN BLOOD IN RAT<sup>1</sup>

By

HARALD TEIR, TAPJO RYTOMAA and ANTTI CEDERBERG

Received 21 63

It has been possible to show that granulocyte production is enormous even in normal conditions, *eg* in man  $\approx 15 \times 10^{10}$  per diem (Craddock 1962). Since in a steady state the elimination must be equal to production, the question that arises is where such a mass of cells is eliminated.

Little is known about the elimination of granulocytes. However, from our present knowledge it can be regarded as obvious that leukocyte elimination occurs chiefly extravascularly (Lissac 1957, Bierman 1961, Craddock 1962). No quantitatively significant number of disintegration forms of granulocytes has been found in the bone marrow and blood in normal conditions (Undritz 1941, Gross 1954a, Petrakis *et al* 1957). Information on the life span of granulocytes and on their blood stream phase (Osgood 1954, Lissac 1957, Craddock 1962), on the ratios of the total granulocyte counts in different organs (Osgood 1954, Donohue *et al* 1958, Rytomaa 1960), and on other kinetics of granulocytes (Craddock *et al* 1959, Finch & Hollnagel 1959, Rytomaa *et al* 1960).

As our several investigators (Gross 1954b, Vashoff 1958, Rytomaa 1960) is that there are surprisingly few disintegration forms of granulocytes in extramedullary tissues especially in view of the enormous number of cells that is produced. This makes it improbable that the disintegration of granulocytes in these tissues could be the main phenomenon.

It seems from the results

of several investigators (Gross 1954b, Vashoff 1958, Rytomaa 1960) who in several

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<sup>1</sup> Sponsored by the Damon Runyon Memorial Fund (DRG 291 F and 644) New York and by the Sigrid Juselius Foundation Helsinki.



The only gross finding of interest at autopsy was enlargement of the heart (wt 120 gm). Microscopic examination, however, revealed focal myocarditis with accumulations of lymphocytes around necrotic muscle fibres. An intracellular, thin-walled pseudocyst, packed with crescent-shaped or round parasites about 3-4 microns long and with distinct nuclei rich in chromatin was also seen. Its appearance was that of a *Toxoplasma* pseudocyst. No inflammatory reaction was seen around the pseudocyst. The groups of lymphocytes in the myocardium probably marked the sites of ruptured pseudocysts.

*Toxoplasma*-myocarditis occurs in both an acute and a chronic form. The prognosis of the latter is much better than that of the former. Cases of myocarditis of unknown origin may be due to *Toxoplasma*. It is, however, difficult to distinguish between *Encephalitozoon*, *Sarcocystis* and *Toxoplasma*.



investigations, using isotope labelling techniques, point to the possible rôle of the lungs as the principal site of granulocyte elimination. According to Bierman, some other routes, *e.g.*, the gastrointestinal canal, urine, and saliva may also play a certain rôle in the physiological elimination of leukocytes. *Pitzurra & Frascarelli* (1948) have assumed that the gastrointestinal canal is the main route of leukocyte elimination. In a more recent study, *Ambrus & Ambrus* (1959) reported that at least labelled lymphocytes were eliminated in the intestinal tract through mucosal diapedesis.

In his review of the "life span" of leukocytes, *Lissac* (1957) concludes that there is little evidence about the routes of leukocyte elimination and that, though the lungs seem to play the most important rôle, they can with reasonable certainty be said only to function as a reservoir of leukocytes. *Burman* (1961), on the other hand, concludes in his review of homeostasis of leukocytes that "it is apparent that the major site of leukocyte destruction is probably extracirculatory, perhaps in the reticulo-endothelial tissues of the lung, spleen, liver, etc."

Several authors have established that the extravascular "reservoirs" of white cells are not easily mobilized (*Osgood* 1954, *Laddlock* 1960). This fact and the observation made in our laboratory, that the gastrointestinal tract contained a very large part of the extramedullary eosinophils in rat (*Rytomaa* 1960), directed our attention to this organ as an important site of granulocyte elimination.

In the present investigation the effect of ileum resection on granulocyte counts in blood has been studied. Though it is clear that this is far from an ideal method to investigate granulocyte elimination, certain information, however, seems quite possible. It should be mentioned in this connection that in reports of the effects of intestinal resections in human, changes in kinetics of granulocytes have not received any special attention.

#### MATERIAL AND METHODS

A total of 109 white male rats of Sprague Dawley strain, aged 3-6 months, were used as test animals. The blood picture of rat can be considered to have reached its final normal level at this age (*Rytomaa* 1960).

The actual method used was ileum resection (colon resection, splenectomy, nephrectomy, and sham operation were selected as control operations). Food was withheld from the rats used for the experiments on the evening preceding the operation. It was allowed again from the last postoperative day. The rats were given water ad libitum throughout. Blood samples were taken from all the animals on the morning of the operation day; the operations were performed in the afternoon under ether narcosis. After the operations the rats were moved in groups of 2-3 into clean cages.

**Intestinal resections.** Laparotomy was done through a fairly long midline incision. Approximately 5 cm from the ileocecal region the ileum was closed with a silk ligature, care being taken not to injure the delicate mesenteric veins. A piece 25-60 cm in length was then resected from the small intestine. The total length of the small intestine in the rats used was ca. 80 cm. Samples were taken from the resected small intestine for histological preparations. Side to side anastomosis was performed using continuous sutures—at first in one layer, later after improvement of the operative technique in two layers.

TABLE 1  
*The Number of Leukocytes and Neutrophils in the Peripheral Blood Before and After Operation, by Experimental Groups*

Group	Before operation			3 days after operation			4 days after operation			9-14 days after operation		
	No. of rats	Leuko- cytes per mm <sup>3</sup>	Neutro- phils per mm <sup>3</sup>	No. of rats	Leuko- cytes per mm <sup>3</sup>	Neutro- phils per mm <sup>3</sup>	No. of rats	Leuko- cytes per mm <sup>3</sup>	Neutro- phils per mm <sup>3</sup>	No. of rats	Leuko- cytes per mm <sup>3</sup>	Neutro- phils per mm <sup>3</sup>
Sham operation	40	14 000	2 480	40	15 800	4 380	29	12 600	2 510	29	17 100	3 170
Splenectomy	10	17 200		10	21 100		10	31 000		10	800	
Nephrectomy	4	10 700	2 240	4	18 600	(3 910)§	2	19 800	(1 700)§	2	(14 800)§	(3 140)§
Colon resection	8	13 900	1 920	8	14 200	5 080	5	14 200	3 900	4	16 500	3 800
Ileum resection	18	16 300	2 460	18	11 300	3 690	12	18 500	4 100	11	19 300	4 950

\* Geometric mean

§ Geometric mean of two samples

## RESULTS

*Neutrophil and Leukocyte Counts in Peripheral Blood*

**Ileum resection** Distinct leukopenia was demonstrable during the first 3 days in the animals on which ileum resection was performed (Table 1 and Figs 1 and 2). Compared with the preoperative leukocyte values the difference was statistically highly significant ( $P < 0.001$ ). Compared with the rats treated with colon resection and other control operations, the leukocyte counts of these animals were again lower ( $P < 0.001$ ).

The absolute neutrophil counts calculated from the differential counts were distinctly lower in the rats with resected ileum than in the other animal groups ( $P < 0.001$ ). The difference was smallest compared with the resected colon animals, but again it was statistically significant ( $P < 0.01$ ) (Fig 2). Compared with the initial values, however, the neutrophil amounts established 1-3 days postoperatively were slightly elevated also in the animals treated with ileum resection (Table 1).

In the rats treated with ileum resection whose blood picture was followed at intervals of ca. 12 hours for the first 3 days, all the changes reported above were demonstrable in the first sample taken after 12 hours. The number of neutrophils, however, was slightly higher in this phase than later.

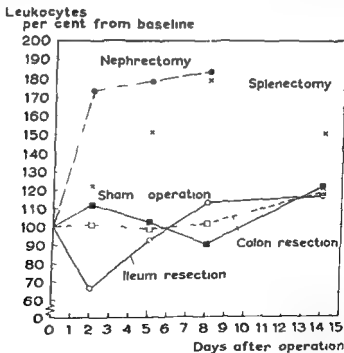


Fig 1

Per cent changes of leukocyte counts in peripheral blood after ileum and colon resections, splenectomy, nephrectomy and sham operation. Preoperative cell counts as baseline.

the first 3 days did not decline compared with the sham operation groups and with the preoperative values (Figs 1 and 2). The transient leukocytosis and leukopenia established in the animals with resected ileum were not demonstrated in these rats.

**Nephrectomy and splenectomy** Pronounced leukocytosis was established in the nephrectomised and the splenectomised animals during the observation time (Table 1 and Fig. 1).

**Sham operations** Manifest neutrophilia was observed in these rats during the first 3 days, but only relatively slight leukocytosis (Table 1 and Fig. 1). Later on the leukocyte values remained relatively even and no leukopenia or transient leukocytosis was demonstrable.

### Eosinophil Counts in Peripheral Blood

As expected, blood eosinopenia was established in the first few post-operative days in all the animals undergoing surgery (Table 2).

New, "spontaneous" eosinopenia was demonstrable in all of the groups on approximately the 7th day. In the majority of the animals this eosinopenia was more pronounced than that established immediately (Table 2).

The eosinophil counts of the splenectomised animals were slightly higher than the initial values except during the eosinopenias seen in the first few days and on the 7th postoperative day (Table 2).

TABLE 2

*The Number of Eosinophils in the Peripheral Blood before and after Operation by Experimental Groups\**

Group	Before operation		1-3 days after operation		5 days after operation		7 days after operation		8-11 days after operation	
	No. of rats	Eosinophils per mm <sup>3</sup>	No. of rats	Eosinophils per mm <sup>3</sup>	No. of rats	Eosinophils per mm <sup>3</sup>	No. of rats	Eosinophils per mm <sup>3</sup>	No. of rats	Eosinophils per mm <sup>3</sup>
Sham operation	30	364	30	232	19	345	19	266	19	376
Splenectomy	10	461	10	356	10	817	10	267	10	502
Colon resection	6	275	6	137	3	3	3	71	3	375
Ileum resection	15	450	15	155	9	188	9	104	8	180

\* Arithmetic mean

† Nephrectomy rats are not included because there were only 2 samples for each

### Total Cell Counts in Bone Marrow

The changes demonstrated in the bone marrow are reported very briefly in the present investigation.

The total cell count in the bone marrow was distinctly higher on the 3rd than on the 2nd postoperative day in the animals with resected ileum. The type of change was similar in those with resected colon, but the total cell counts were even below control values (Fig. 3).

The leukocyte values of individual animals began to show considerable variation after the slight increase in the number of leukocytes established around the 9-10th day. The increases and decreases seemed to happen independently and did not follow any demonstrable rhythm.

Transient (duration shorter than 1-2 days), very pronounced leukocytosis was demonstrable at a later phase in two-thirds (8/12) of the animals with resected ileum. The leukocyte counts generally rose to 50 000-60 000 per  $\text{mm}^3$ , the highest value established was 76 750. Concurrent eosinophilia and absence of the shift to the left in the differential count was observed during these leukocytosis attacks. The general good condition of the rats was unchanged during the leukocytosis attacks. Rats with obvious infection (not included to the final material) showed distinctly different changes in the blood picture, i.e., eosinopenia and shift to the left in the differential count. Furthermore, the leukocytosis in rats with obvious infection was much less pronounced than in rats with "transient leukocytosis".

Two thirds (7/12) of the rats surviving for longer period developed pronounced, acute leukopenia (not preceded by leukocytosis), sometimes with nearly total agranulocytosis, during which they died rapidly.

*Colon resection* In the animals with resected colon, on contrast to those with resected ileum, the leukocyte and neutrophil values during

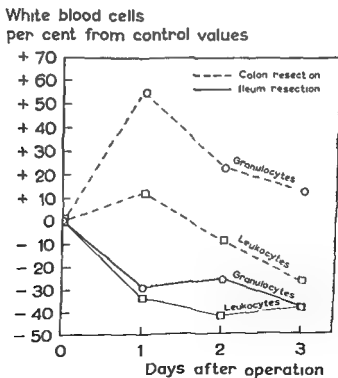


Fig 2

Per cent changes of neutrophil and leukocyte counts in peripheral blood during the first 3 days after ileum and colon resections. Cell counts of sham operated animals as baseline

TABLE 3

*The Number of Erythrocytes in the Peripheral Blood after Operation  
some of the Experimental Groups*

Group	Under two weeks after operation		Over two weeks after operation	
	No. of rats	Erythrocytes (in millions) per mm <sup>3</sup>	No. of rats	Erythrocytes (in millions) per mm <sup>3</sup>
Sham operation	6	6.8	8	6.9
Colon resection	5	7.0	5	6.5
Ileum resection	7	6.0	7	5.9

\* Arithmetic mean

### *Weight of the Animals*

The weight of the test animals fell sharply—ca. 20 per cent—in the resected ileum group during the first 2 weeks, in the resected colon group less—ca. 10 per cent—and in the sham operated group not at all. The weights of the resected ileum animals generally did not return to the initial value, not even at a later stage (Table 4).

TABLE 4

*The Weights of the Animals before and after Operation  
some of the Experimental Groups*

Group	Before operation		Under two weeks after operation		Over two weeks after operation	
	No. of rats	Weight in grams*	No. of rats	Weight in grams*	No. of rats	Weight in grams*
Sham operation	8	240	6	240	8	250
Colon resection	8	235	5	210	5	220
Ileum resection	7	220	7	175	7	195

\* Arithmetic mean

### DISCUSSION

There are reports in the literature on the possible significance of the gastrointestinal canal for the removal of leukocytes. In numerous older studies with different animals granulocytes have been found in histological preparations in the gastrointestinal tract between the mucosal cells and sometimes even in the lumen (Weil 1920, Waxman 1927, Hamperl 1932). Pilzura & Frascarelli (1943) examined 10 persons for the total cell quantities in the urine, saliva, gastric juice, bile, and faeces and from the values obtained in the counting chamber and smears calculated the number of leukocytes eliminated per 24 hours along these routes. The results showed that the gastrointestinal canal was probably the principal site of leukocyte elimination. Only very few



Total  
number  
of cells  
 $\times 10^6$

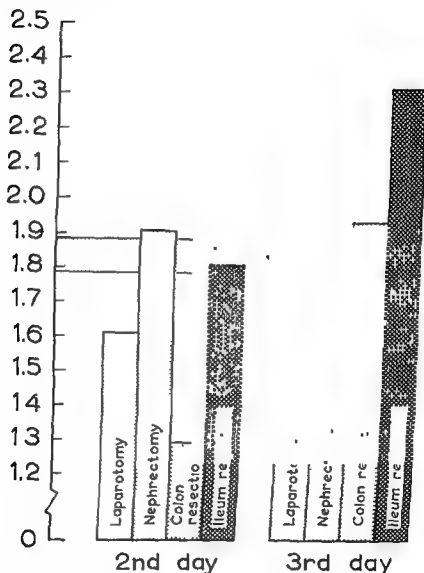


Fig 3

Total cell counts per mg of bone marrow on the 2nd and 3rd day after ileum and colon resections, nephrectomy, and sham operation (cell counts (range) of 4 untreated male rats of the same population as baseline)

### *Erythrocyte Counts in Peripheral Blood*

Throughout the observation period the erythrocyte values of the rats with resected ileum and colon did not differ significantly from values of animals given a sham operation (Table 3)

days) and the unchanged general good condition of the rats could be interpreted as phenomena against infection. Furthermore the changes in blood picture in rats with obvious infection were quite different from these leukocytosis attacks. Thus it seems possible that the causative mechanism could have been a disturbance in the elimination of granulocytes.

Severe leukopenia which at times approached complete agranulocytosis was demonstrable upon the death of the animals and sometimes even at other times in rats surviving ileum resection for more than 2 weeks. The differential count often revealed a distinct shift to the left which can be regarded as an indication of the absence of a serviceable granulocyte reserve. However the absence of a marrow granulocyte reserve was not necessarily due to exhaustion of granulocyte production since it was later observed in other series of experiments that the total number of myeloid cells in the bone marrow was not decreased. The observation could be explained by a maturation arrest (malnutrition?) or alternatively by a compensatory inhibition of granulocyte maturation because of the block in peripheral elimination. About two thirds of the rats died during the leukopenia of an obvious secondary infection.

The results of the present investigation lend support to or at least do not disagree with the hypothesis that especially the small intestine has an important role in the elimination of granulocytes. However according to the recent literature there seems to be some general agreement on the possible significance of the lungs in the removal of granulocytes (Bierman *et al* 1955, 1959, Issac 1957). It is obvious that granulocytes in relatively numerous amounts even can be found in the sputum but to the best of our knowledge the quantitative importance of this

he mentioned

cytes produce

conditions in volume to ca 70 ml (if 1 ml is equivalent to  $2 \times 10^9$  granulocytes (Tivey *et al* 1951))

According to the observations already referred to in the introduction it seems very probable that the disintegration of granulocytes in the bone marrow, blood and in most extramedullary tissues could be the main

(Issac 1957) that the amount of granulocytes removed in 24 hours via the saliva and oral mucosa, urine, skin and serous membranes seems to be practically insignificant compared with their production.

According to the available literature it seems evident that granulocyte elimination takes place in extravascular tissues (Issac 1957, Bierman 1961, Craib 1962). It follows that either the granulocyte turnover is rapid in that organ which plays the most important role in

more recent investigations, using radioisotope labelling techniques, suggest the intestinal tract as a possible site of leukocyte elimination (Bierman *et al* 1955, Ambrus & Ambrus 1959, Astaldi 1960), and they do not cite any quantitative values. Ambrus & Ambrus (1959), however, reported that at least labelled lymphocytes were eliminated in the intestinal tract through mucosal diapedesis.

Our interest in the kinetics of granulocytes was aroused by investigations on eosinophil granulocytes (Rytömaa 1960). These studies established that in rat eosinophils are ca. 200 times more numerous both in the bone marrow and the extramedullary tissues than in the blood. Especially the very large proportion of extravascular eosinophils in the gastrointestinal tract in rat directed our attention to this organ as a possible principal site of granulocyte elimination.

To test the validity of this assumption, we began by studying the effect of resection of the intestine on the granulocyte counts in the blood, bone marrow, and in various tissues. It is clear that the method used in the present investigation may have caused many nonspecific effects. As the quantity of granulocytes in peripheral blood in particular is influenced by numerous factors, some changes established concurrently in the cell counts of the bone marrow were considered briefly. Furthermore, on the basis of our direct observations on granulocyte elimination in the intestinal tract it seems quite probable, that the results obtained in the present work warrant certain conclusions regarding the specific role of the intestinal tract on the kinetics of granulocytes.

The investigation established that ileum resection leads to leukopenia and, compared with the other operations, including colon resection, neutropenia lasting for ca. 3 days. On the third day after ileum resection the bone marrow cell counts, by contrast, were increased to above the normal level although they had been normal on the preceding day. The decreases in the total of cells in the bone marrow on the second day were obviously caused by the mobilization of the marrow granulocytic reserve (Cradlock *et al* 1960), the increase on the third day probably by the stimulation of production. Alternatively, the increase on the third day could be explained by an inhibition of granulocyte release from the bone marrow, probably because of a block in the peripheral elimination mechanism. Similarly, the increase in leukocyte values in peripheral blood occurring ca. 9-10 days after ileum resection suggests stimulation of cell production in the bone marrow or, alternatively, inhibition of cell elimination in the periphery.

Pronounced attacks of leukocytosis of very short duration in which the number of cells generally increased to values approaching leukemoid reaction were established in two thirds of the rats undergoing ileum resection whose blood picture was followed at 1-2 day intervals. The concurrent eosinophilia, the absence of the shift to the left in the differential count, the short duration of the reaction (shorter than 1-2



granulocyte elimination, or the organ must be large. Thus, bearing in mind the generally accepted view of the defensive function of granulocytes, it is not unlikely *a priori* that the intestine has an important rôle in the elimination of granulocytes.

### SUMMARY

Resections of the small and large intestine, splenectomy, nephrectomy, and sham operation were performed on 109 male rats in order to study the effect of these operations on the granulocyte counts in the blood. The final material included 80 of these rats, since animals with obvious infection were excluded.

A distinct leuko- and granulocytopenia in the peripheral blood was demonstrable during the first 3 days after the ileum resection. In the other groups, the usual postoperative leuko- and granulocytosis occurred.

In the rats with resected ileum the leukocyte values of individual animals showed considerable variations from the 9-10th day. High, transient (under 1-2 days) leukocytosis approaching leukemoid values was established at a later phase in two-thirds of the animals. The leukocyte counts generally rose up to 50 000-60 000 per cu mm. No evidence of infection was noted in the blood picture, the general condition of the rats remained unchanged. Two-thirds of the rats surviving for 3-4 weeks or more developed acute marked leukopenia—independent on leukocytosis attacks—during which they died rapidly. Leukocytosis and leukopenia of this kind did not occur in the colon resection or other experimental groups. A noteworthy leukocytosis was established in the nephrectomised and the splenectomised rats.

A "spontaneous" eosinopenia on the 7th day was observed in all experimental groups in addition to the usual postoperative eosinopenia. There was no change in erythrocyte counts.

A preliminary study of the total cells counts in the bone marrow showed a distinct increase on the 3rd postoperative day in the rats with resected ileum.

The present investigation lends support to, or at least does not disagree with, the hypothesis that the intestinal tract has an important rôle in the physiological elimination of granulocytes.

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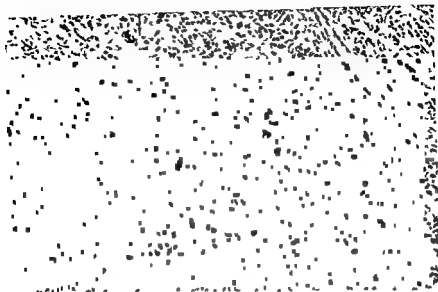


Fig 1

Biopsy taken 1941 from intumescence above the left orbit. The diagnosis was  
 Sympathicoblastoma H & F  $\times 120$

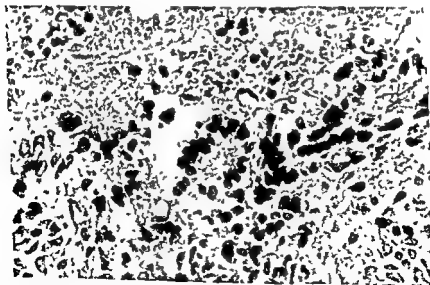


Fig 2

Higher magnification of a rosette from the slide shown in Fig 1 H & F  $\times 384$

## TRANSFORMATION OF SYMPATHICOBLASTOMA INTO GANGLIONEUROMA

*With a Case Report*

*By*

JAKOB VISTFJELD

Received 10.1.63

In recent years clinical and experimental studies on embryonal tumours in children have appeared with increasing frequency, tumours apparently derived during the organogenesis from primarily non differentiated, multi potent cells. Such cells have been assumed to retain their power of differentiation and it has been suggested that a differentiation might be therapeutically induced thus provoking a conversion into less immature benign tumours presenting reduced growth energy. This theory, however, is based on clinical reports of a few cases only in which such conversion actually had been observed, mostly cases of sympathicoblastomata which, whether treated or not, converted into latent ganglioneuromata composed of apparently mature ganglion cells. Only few of these reports, however, are based on histologically convincing data.

The here discussed case has been described on two previous occasions, viz. as case no. 2 in a study from 1942 by *Thomas Rosendal* (12) and as case no. 1 in a study from 1953 by *Poul Bjerre Hansen* (8). A metastasizing sympathicoblastoma originating assumedly from the adrenal cortex, had reverted in the course of intense radiotherapy and presented no signs of a further activity. In 1962 the patient died from an intercurrent disease and was autopsied, it was realized that this was one of the rare cases in which a metastasizing sympathicoblastoma had "matured" into a highly differentiated ganglioneuroma. Available histological data are published here together with a summary of the case history. Theories concerning this type of tumour and clinical therapeutic endeavours are discussed.

### CASE REPORT

(Summed up on the basis of reports by *Rosendal* (12) and *Hansen* (8) together with the case record no. 77944 from the Radium Centre in Copenhagen)  
A boy born on February 23 1941. Disease set in in August 1941 the initial symp

directly distally to the adherence a pea sized perforation was noted from which a biopsy was taken. The posterior wall probably also the pancreas were involved. A biopsy was taken also from the posterior wall. The histological examination of the biopsies showed a presence below the glandular tissue of a strange tissue rich in nuclei, much reminding of connective tissue formation with densely arranged irregular occasionally large and dark nuclei. In the histological diagnosis the hypothesis was voiced that it might be tumour tissue.

Hence the patient was referred to the Radium Centre in Copenhagen and on the not too well founded suspicion of recurrent tumour activity moderate doses of roentgen irradiation were given to the upper abdominal region totalling 1500 r to the anterior field 1450 r to the posterior field. At his discharge from hospital the patient felt well.

On June 26 1962 however haematemeses occurred and the patient was admitted to the medical department of the Central Hospital in Randers. During his stay there haemorrhages were severe haematemeses as well as melaena assumedly attributable to an ulcer with ingrowth of tumour tissue. The surgeons were consulted and it was deemed ill advised to institute surgical measures consequently the patient was treated by roentgen blood transfusion saline and glucose solutions. Terminally the patient was running a high temperature and death occurred on July 16 1962.

Autopsy performed by the author of these presents took place on the following day. Data of the strange course of disease were sparse at this stage. The gross findings included extreme paleness, some irregularity of the calvarial bones, slight protrusion of the eyes which, however, were not displaced in their sockets. No lymph nodes were palpable.

The pleural cavities on either side contained 1 liter a clear, slightly yellowish fluid and fibrinous membranes lined the pleural laminae. The lungs presented stasis and oedema, dispersed minor haemorrhages, and numerous, delicate bronchopneumonias.

Lining the trachea and below the bifurcation a number of enlarged lymph nodes were observed, the diameter of the largest being 3 cm. A few lymph nodes were localized to the neck. All were well-defined, in sections of a greyish, completely uniform, tough, and rather soft consistency.

Gross inspection of the stomach and duodenum disclosed no tumour infiltration but distally by 15 cm to the pylorus a typical, 3 × 2 cm wide ulcer was demonstrated the bottom of which was of a greyish colour, necrotic, presenting a prominent process with a large vessel. The ulcer invaded the entire wall, its bottom being in close apposition to the pancreas. No tumour tissue was demonstrable by gross inspection of the ulcer. Blood and melaena were abundant in the intestines.

The liver and the bile ducts were natural, no metastases were observed, and also the pancreas was natural, the spleen weighed 250 g presenting the features of a typical, infectious spleen.

The left suprarenal gland was natural. In the retroperitoneal space, at the site of the right suprarenal gland, a large whitish tumour was demonstrable (Fig. 3), it had no actual capsule but, being encircled by connective tissue, it was rather well-defined. Its size was about 10 × 10 × 5 cm, the weight 250 g, the tumour was in close apposition to the uppermost, right, renal pole, its position being rather medial to the



toms including extravasation of blood in the eyelids frontotemporal swellings and some protrusion of both of the eyeballs. The child was admitted to the paediatric department of the State Hospital (*Rigshospitalet*) where roentgen examination of the skull disclosed a radiating spicule formation in the frontotemporal intumescences. Numerous small rarefactions were seen laterally in the frontal bone and in the right mandible. Otherwise no skeletal anomalies were demonstrable. The bone changes were interpreted—with certain reservations—as haemangiomas. A large abdominal intumescence was palpable, extending from the right curvature as far down as to the right flank. Roentgen examination of the abdomen showed at the site of the right kidney, a soft-tissue shadow, its size being as a half grape fruit. Numerous small calcifications were observed. A biopsy was taken from the intumescence above the left orbit, and a histological diagnosis was established as sympathoblastoma (Figs 1 and 2) (*G. Teitum*).

Roentgen irradiation was given to both temporal regions and to the tumour to the right in the abdomen, totalling 600, 900, and 950 r to each field. During and after treatment the general condition of the patient improved. The intumescence above the orbits flattened, the exophthalmus subsided, the abdominal tumour was found to have regressed a little on the right side but was now extending a little to the left of the first lumbar vertebra. Roentgen irradiation was repeated a few months later with somewhat lower total dosages.

In 1946 the patient was admitted to hospital again, this time on account of some strange prominences developing in the skull, particularly in the right parietal region where the cranial wall by roentgen examination was found to be very thin. Sclerotic areas and some osteolytic spots were noted on either side in the orbital and temporal parts of the frontal bone, but no development of spicules.

The prominences in the skull were exposed to serial roentgen irradiation the dosage totalling 1800 r to three fields. The general condition of the patient was satisfactory but gradually his sight failed and a bilateral optic atrophy developed.

In 1951 the patient was re-admitted to hospital, first to the neurosurgical department but later he was referred to the Radium Centre in Aarhus. In all essentials the cranial bone changes remained stable, sclerosed areas with occasional rarefactions were observed around the orbits. In addition isolated small sclerosed spots and rarefactions were seen in some of the ribs, in the left scapula, in some of the vertebrae, and in the pelvis. The tumour had displaced downwards and anterolaterally the right kidney, the left kidney was in normal position. The symptoms were interpreted tentatively as tumour of the suprarenal gland with sclerosed

prevailed as regards the character of  
onally might be rather tender, a total  
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sue was found to be of a soft spongy

non characteristic nature. Histologically it was found to be composed of connective tissue with only few cells and vessels but symptoms were not demonstrable of active pathological processes (*W. Munk*). Radiotherapy was discontinued.

Development of the patient was satisfactory in the following years, he came under the care of the blind. His intelligence seemed to be normal and his physical development was normal.

In 1956 the left lobe of the thyroid gland was found to be the site of an adenomatoid smooth swelling, it could hardly be a metastasis from the sympathoblastoma which remained completely inactive. Extirpation however was found advisable and a left-sided hemistrulectomy was performed in Department K of the State Hospital. The histological examination showed the presence of a cellular follicular adenoma of the thyroid gland (*Hemming Poulsen*).

During the following years the patient met for control at frequent intervals at the Radium Centre in Copenhagen. In all essentials the condition seemed to be satisfactory.

In January 1962 the patient felt diffuse abdominal pain and was admitted to the local hospital where the diagnosis acute abdomen was established. On the day of admission the patient was operated, a perforated gastric ulcer was sutured and appendectomy was performed.

The gross appearance of the appendix was normal. Behind the gallbladder displacing the latter to the fore and the kidney in a downward direction, a retroperitoneal immovable rather bulky tumour the size of a fist was found. In an area of about 2 cm the anterior gastric wall adhered to the anterior abdominal wall.

latter. Consequently the right kidney had been slightly displaced in a downwards and lateral direction. In the upward direction the tumour extended to the hilum of the liver without being connected with the bile ducts but closely adjoining the enlarged lumbar lymph nodes. The entire tumour and the surrounding connective tissue were easily detached, they were completely independent of the gastro intestinal system. Upon isolation of the tumour the right suprarenal gland was found to be embedded in the loose connective tissue encircling the tumour. Gross inspection presented a normal suprarenal gland, there was no continuity between the suprarenal gland and the tumour. In sections (Fig 4) the tumour and the lumbar lymph nodes presented a consistency of a rather uniform, whitish, rather whirled, solid, and tough character. No soft areas were noted, nor haemorrhages and large calcifications.

The weight of the left kidney was 140 g., of the right only 80 g., the latter being slightly deformed. The capsules were easily detached, surfaces were smooth, and the parenchyma was normal.

The spine was opened using a chisel and an about 1 cm large, grayish, rather soft focus of a metastatic aspect was found centrally in the body of the VIII thoracic vertebra. The skull was opened and the brain excised. Gross inspection disclosed no anomalies in the brain, cerebellum, pituitary gland, pons, and medulla oblongata. Also the cranial bones were opened with a chisel, *e g* both orbits, but no metastases were demonstrable. The two optic nerves were dissected but pathological processes were not seen. The prominence of the dorsum sellae was rather remarkable but normal bone tissue exclusively was seen when it was opened with a chisel.

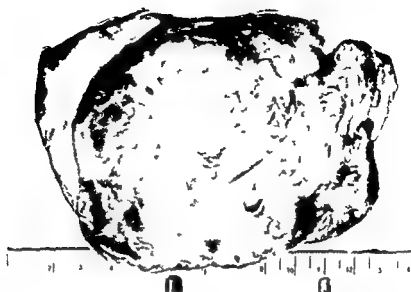
Specimens for microscopical examination were excised from various parts of the tumour, from the metastatic thoracic and lumbar lymph nodes, from both of the suprarenal glands, the lungs, spleen, and kidneys. A small bone specimen from the dorsum sellae and one from the occipital bone were sent for decalcification but have been lost later.

### *Slides Prepared Previously*

Microscopy of biopsy taken in 1941 from the intumescence above the left orbit, labelled KP 1885 41—Figs 1 and 2

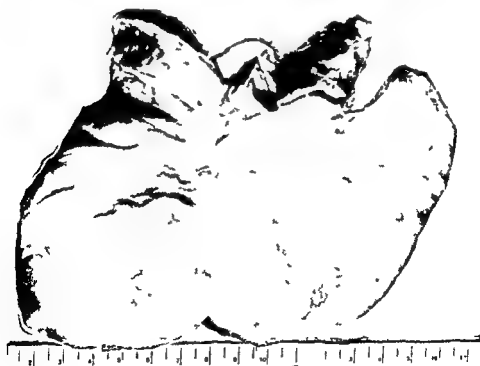
The tumour	brils
of the stroma	the
tumour cells	are
immature, spheric	with rather small, hyperchromatic nuclei and sparse cytoplasm. Some are larger, their nuclei more loose and the cytoplasm more abundant. The tumour cells occur isolated or may be seen arranged in short chords, occasionally forming characteristic ro

<sup>1</sup> Professor G. Teislum MD has kindly provided supplementary sections from the block for re-examination.



*Fig 3*

Tumour (ganglioneuroma) removed at autopsy 1962 from the retroperitoneal area above the right kidney



*Fig 4*

Cut surface of the tumour shown in Fig 3



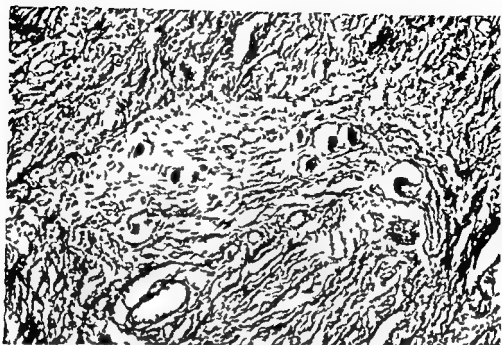


Fig. 5

Section of the ganglion neuroma seen in Figs. 3 and 4. Neurofibril staining  $\times 1$

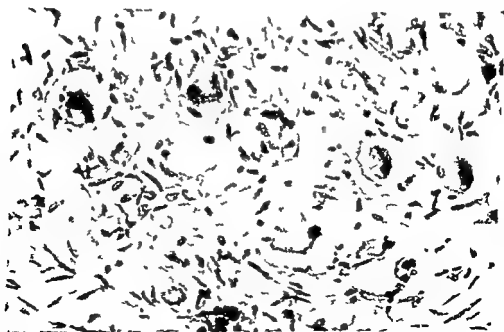


Fig. 6

Section of the ganglion neuroma shown in Figs. 3 and 4. Area with collections of ganglion cells. Gallocyanin chromalum  $\times 300$



Fig 7

Section of metastatic thoracic lymph node removed at autopsy  
Gallopanchromalum  $\times 181$

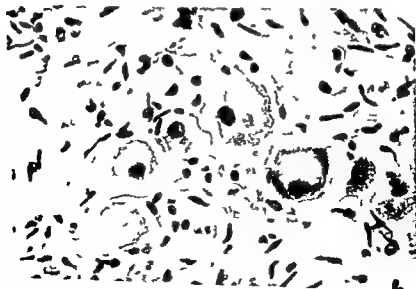


Fig 8

Detail of Fig 7  $\times 480$



Fig 5

Section of the ganglioneuroma seen in Figs 3 and 4 Neurofibril staining  $\times 120$

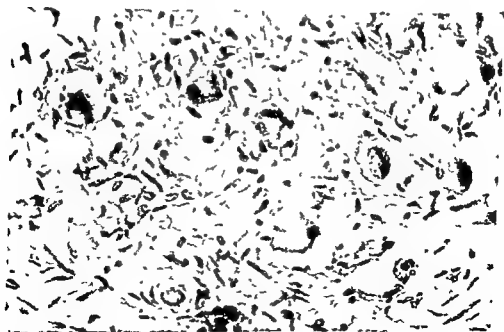


Fig 6

Section of the ganglioneuroma shown in Figs 3 and 4 Area with collections of ganglion cells Galloevanin chromalum  $\times 300$

emergency hospitalization is required and, operation performed immediately upon admission, disclosed a perforated gastric ulcer. On this occasion the large abdominal tumour is observed. A biopsy is taken from the edge of the ulcer. Microscopy of the biopsy suggests some tumour ingrowth into the stomach.—At re-admission 3 months later on account of a bleeding ulcer, the institution of surgical measures is found inadvisable and the patient is treated conservatively, death occurs 3 weeks later. Autopsy revealed the presence of a large duodenal ulcer, in the retroperitoneal space a well defined ganglioneuroma is found together with metastases of similar tissue to the lumbar, thoracic, and cervical lymph nodes, in addition a small focus is noted in the VIII thoracic vertebra, metastases to the skull are not demonstrable. Diagnosis established on the basis of the histological examination of the tumour metastases to the lumbar and thoracic region present uniform ganglioneuroma tissue. Areas presenting neuroblastoma tissue are not demonstrable.

#### DISCUSSION

Neuroblastoma is the solid tumour most frequently found in infants and young children (5). Generally the tumours are diagnosed within the first year of life and may admittedly develop even prenatally (1). Most of the patients die very soon after birth but in rare cases the tumour has been seen to disappear or to "mature", i.e. the malignant cells convert into highly differentiated ganglion cells. Hence, as observed by *e.g.* Willis (18) in embryologic studies, the parallelity of developments of tumours and normal ganglia seems to be very close. The latter are derived probably from the neural crista and are found primarily as formations of densely arranged round cells, the cells may be arranged in parallel cords although the characteristic rosettes with the central delicate network of fibrils represent the most specific finding. The mature ganglion cells and nerve fibres develop from these structures (18). Development of all of the ganglia in the autonomous system is not completed until the age of about 10 to 12 years has been attained. Up to this age the incidence of neuroblastomata has gradually abated. Generally the histological features of neuroblastomata are characteristic, including the densely arranged round cells with formation of many rosettes or signs of the latter. It should be expressly emphasized that although formation of rosettes represents a characteristic, morphologic feature, it need not always be demonstrable in the rapidly growing tumours and hence, it does not represent a necessary criterion on which to establish a diagnosis, these deliberations are in accordance with the above discussion of the development of ganglia.

Lushington & Wolback (3) were the first who in 1927 reported a histologically verified case of malignant paravertebral sympathicoblastoma which had converted into a benign ganglioneuroma. The follow up of



settes or their approximates. No ganglion cells are observed. The picture is characteristic of a *sympathicoblastoma*.

Slides (labelled 587/62) of biopsies, taken in January 1962 on the occasion of the operation for perforated gastric ulcer, have been studied. Re-examination of these slides failed to disclose a presence of tumour cells.

### *Autopsy Slides*

*Tumour* (Figs 5 and 6) The appearance is perfectly uniform in the various areas. The tumour is composed of fibrillary tissue arranged in undulating strands and bundles. Dispersed accumulations of isolated, highly differentiated and polymorphous ganglion cells are seen. They may be ovoid or polygonal, and characteristic axons and dendrites are seen, many of the cells contain a brown pigment, in a few the nucleus is eccentrically arranged or the nucleus may be large and nodular, some present 2, 3, or 4 nuclei. In the predominating, fibrillary tissue are some fibroblasts, but other cells may also remind of Schwann's cells. Occasionally the nerve fibres are collected into circular structures as known from peripheral nerves. Vascularization with delicate capillaries is abundant. Numerous small calcified foci and some fatty infiltration are seen. But tissue reminding of neuroblastoma is not demonstrable. The picture is characteristic of a *ganglioneuroma*.

*Metastatic thoracic lymph nodes* (below the bifurcation of the trachea, Figs 7 and 8) Features are similar to the ones described above. The presence of some accumulations of lymphocytic cells suggests that it may be a matter of metastases to lymphatic tissue.

*The suprarenal glands* Cadaverosis is vivid but otherwise structures are normal.

*Duodenal ulcer* The presence of a typical, large, chronic, duodenal ulcer is demonstrated, lined on either side by Brunner's glands. The ulcer invades the muscles, a small zone of fibrous tissue covering the underlying pancreatic tissue. No tumour cells at all are demonstrable.

### I P I C R I S I S

A 20-year-old man is admitted to hospital with acute haematemesis. When the patient was 6 months old the presence of a large tumour in the right renal region had been ascertained, metastasizing particularly to the skull. A biopsy taken at that time from the intumescence above the left orbit suggested the presence of a *sympathicoblastoma*. Intense roentgen irradiation to the abdomen and the skull had been administered upon which no further growth of the tumour occurred. roentgen examination had verified the development of delicate calcifications in the tumour. The cranial metastases retrograded. An optic atrophy and reduced sight represented the sequelae. Otherwise the development of the patient had progressed normally. —In January 1962

which not tumour originally had been demonstrated together with path . . .

and the metastases in the skull 20 years later the patient dies from a bleeding duodenal ulcer, and the large tumour in the right renal region is demonstrated at autopsy. It should be noted, however, that metastases were found also in *e.g.* the thorax to which no irradiation had been given. In all of the specimens excised from the tumour and the metastases the presence of ganglioneuroma tissue is demonstrated exclusively, no neuroblastoma tissue.

**Therapy** Various measures have been introduced in the treatment of neuroblastomata and the results obtained have given some reason to optimism. These therapies, isolated or combined, include (20) 1) surgical, radical excision of the tumour, 2) roentgen irradiation, the therapeutical doses generally ranging from 1500 to 3000 r, 3) chemotherapies, previously using Coley's toxin, aminopterin, amino anfol, etc., lately particularly using B<sub>12</sub>. With a view to illustrating the results obtained the following studies shall be briefly referred. Wittenborg (19) reported a material including 73 cases, 3 of the 28 non-treated patients (10 per cent) survived for 3 years or more. 45 of the patients were treated by a) surgical measures, b) surgical measures combined with postoperative roentgen irradiation; or c) roentgen irradiation exclusively. 30 per cent of the total survived for 3 years or more. In 60 per cent of the cases "radical" extirpation of the local tumour resulted in survivals for 3 years, 60 per cent of the cases in which extirpation had not been radical but roentgen irradiation had been given, survived for 3 years. Phillips (11) published a material collected from several hospitals totalling 155 cases, 22.6 per cent presented a 3 year survival or above. Horn *et al.* (9) reported 44 cases, 41 of the patients, follow-ups covering 14 months, included 16 patients who survived and became symptomfree and 25 patients who died. These authors advocate the institution of surgical measures as radical as possible. Uhlmann & von Bassen (17) report a material including 20 patients, all of these were treated by roentgen irradiation. In 7 cases the time of survival ranged from 22 months up to 13 years. In all 7 cases the administration of roentgen had been based on biopsies. In 5 of these cases metastases had been present at the time of treatment. Reports are available, as already mentioned, of a few patients who had received Coley's toxin, probably without benefit (3, 4, 16). Recent studies by Bodian *et al.* (2) are of much greater interest. Here the results from administrations of large, long term doses of B<sub>12</sub> are reported although it must be admitted that the patients had received additional treatment by conservative measures. The material includes 72 children who all received the drug, 7 of these were followed for less than one year. Regression of the tumour was seen in 28 of the cases—2 of these children are still alive and, with one exception, have been followed for one year or more, 2

the patient had covered 16 years. This case has been repeatedly discussed; the patient has been re-examined and the case has been reported again (together with another case) *e.g.* by Fox *et al.* in 1959 (4). Reports of several other cases are available (*e.g.* 10, 11, 16, and 17) but few of these are substantiated by reliable histological data. Reports are abundant, however, of cases in which regression is total.

In the course of regression or conversion of neuroblastomata into ganglioneuromata, whether spontaneous or during treatment, the initial symptoms generally include: haemorrhages, necroses, and small calcifications. Occasionally this process may be verified by roentgen examinations. Changes in the tumours need not be uniform and, according to experience, the appearance of the tumours, macroscopical as well as microscopical, may be highly different in the various areas including also the intermediate stages. The histological diagnoses most commonly established: neuroblastoma—ganglioneuroblastoma—ganglioneuroma—give evidence hereof. The theory that ganglioneuromata always develop from neuroblastomata seems obvious (14, 15). It has been mentioned already that neuroblastomata occur mostly in children although a few reports are available of developments later in life, Halpert *et al.* (7) *e.g.* found a metastasizing neuroblastoma in a 53-year-old patient, and Willis (18) describes a neuroblastoma developing in a 51-year-old patient. The explanation is probably that neuroblastoma elements in a ganglioneuroblastoma for unknown reasons have become stimulated to renewed growth and metastasization. According to expectation, ganglioneuromata become manifest later in life than neuroblastomata. Some are prone to give symptoms in childhood but many remain unnoticed until adult age and may not be diagnosed until the patient appears for a routine roentgen examination. Reports are available of cases even in which such tumours represented casual autopsy findings in middle-aged or old patients (18). In fully differentiated, benign ganglioneuromata nothing but mature nerve cells and nerve fibres are demonstrable. Shapes of the cells may be ovoid, polygonal, or pyramidal like normal or degenerated ganglion cells. Not infrequently they contain a brownish pigment. The fibres are arranged in broad strands and bundles with or without Schwann's cells. Most, if not all, ganglioneuromata contain vast amounts of fibres with rather few and dispersed groups of ganglion cells. But so far it remains to be elucidated why this is so. In the rare cases of metastases or invasive growth from tumours histologically presenting the features of ganglioneuromata the diagnosis, malignant ganglioneuroma has been suggested (*e.g.* Uhlmann & von Essen, case no. 18 (17)). Still, mitoses are probably never present and the ganglion cells are of completely the same type as the ones encountered in the so-called benign ganglioneuroma. In my opinion these rare cases should be interpreted as originally metastasizing neuroblastomata the primary tumour of which together with the metastases have converted, a theory which is substantiated by findings from the here discussed case in

## SUMMARY

In 1941 a 6 month old child was found to have a large, para vertebral tumour and multiple metastases particularly to the skull

of intense roentgen irradiation to the skull and the abdominal tumour the cranial metastases regressed and calcifications developed in the abdominal tumour. The development of the patient progressed normally, but in 1962 he died from a bleeding duodenal ulcer. At autopsy a well defined retroperitoneal tumour was found together with metastases containing similar tissue in the lumbar, thoracic, and cervical lymph nodes, in addition a small focus was noted in the VIII thoracic vertebra. Metastases to the skull were not encountered. Histological examination of the tumour and the lumbar and thoracic metastases disclosed the presence of uniform ganglioneuroma tissue. No neuroblastoma tissue at all was seen. The question is discussed whether differentiation may be therapeutically induced in cases of neuroblastomata. On the basis of previously reported studies the various therapeutical measures are discussed including operation, irradiation, and chemotherapy. Even widespread metastases do not contra indicate the application of therapeutical irradiation since regression or "maturing" of metastases occasionally may be obtainable even if they are not hit by the irradiation. The here discussed case is in support of this contention.

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of the children have died from intercurrent diseases. For many reasons the results cannot be readily compared. In general it may be concluded that operation always is indicated provided that it can be sufficiently radical, indeed it must be admitted that satisfactory results have been obtained also in cases in which the operation had not been radical but rather been combined with postoperative roentgen irradiation, and results have been satisfactory even in cases treated by roentgen exclusively. The value of the  $B_{12}$  therapy remains to be elucidated. In conformity with other investigators (17) some characteristic features should be emphasized. It can hardly be denied that some of the tissue invaded by tumour cells has escaped irradiation in surviving, roentgen treated patients. Hence, irradiation of certain areas of the tumours seems to provide regression or "maturing" even in some of the non-irradiated areas. The case here discussed serves as example. Widespread metastases do not represent a contra-indication to intense radiotherapy.

Being aware of the proneness of malignant embryonal tumours to differentiate and turn inactive, expectations have been great as regards the practicability of inducing therapeutically such differentiation. The problem is fascinating although it should be borne in mind that, for all practical purposes, our insight into factors inducing spontaneous "maturing" is almost non-existent. Hence, the therapeutical endeavours have been based on empiricism. During roentgen irradiation neuroblastomata have been seen in rare cases to convert into ganglioneuromata but this finding should be taken with a certain reservation, always remembering that tumours are prone to spontaneous "maturing". Occasionally the roentgen irradiation may involve a direct cancerocidal effect (17). So far it remains to be elucidated whether or not such effect is involved also in the treatment using  $B_{12}$ . If conversion into benign types occurs during treatment it would be rather unjust, on the basis of our present insight, to explain this feature as a direct effect of the therapeutical measures. Until otherwise demonstrated it seems obvious that irradiation (possibly also chemotherapy) may exert a certain cancerocidal effect on the most immature of the tumour cells and hence allow the remaining, "surviving" cells to pass through the process of differentiation which admittedly embryonal tumour cells do spontaneously. The differentiation of embryonal tumours should be further examined, clinically as well as experimentally. Neuroblastomata as well as ganglioneuromata may develop in animals (6, 13) and experiments using transplanted "mouse neuroblastomata" are in progress (2). Goldstein & Pinkel (5) professed to having demonstrated a differentiation into ganglionic cells using long term cultures with human neuroblastoma tissue. Uhlmann & von Essen (7) emphasize the demand for histological follow-up studies of surviving patients — A further examination of the biological features of these tumours is required if ever we shall have hopes of arriving at a more effective therapy.

## THE THYROGLOBULIN POOL IN THE THYROID GLAND IN PATIENTS WITH AND WITHOUT THYROID AUTO ANTIBODIES

By

TAGE HJØRT

Received 23 XII 62

During recent years numerous investigations have shown that auto immune reactions occur very frequently in a variety of thyroid disorders (Roitt & Doniach 1958 1960 Paine *et al* 1957, Blizzard *et al* 1959 Mackay & Perry 1960) Three different types of thyroid specific auto antigens are now known The best studied of these is thyroglobulin (Doniach & Roitt 1957 Witebsky *et al* 1958), which normally occurs only in the colloid of the thyroid gland However the colloid also contains another auto antigen—the second colloid antigen (CA 2)—the nature of which is as yet unknown While the thyroglobulin antibody can be demonstrated by precipitation the tanned cell technique and Coons technique (CA 2 antibody can only be revealed by Coons technique (Balfour *et al* 1961) The third antigen is found within the cells in the highest concentrations in the glandular tissue of thyrotoxic patients (Roitt & Doniach 1960) The corresponding antibody is usually demonstrated by a complement fixation test ( Complement fixing antibody ) The cytotoxic effect produced by certain Hashimoto sera when these are added to fresh cultures of trypsinized thyroid cells has been ascribed to a fourth antigen antibody system (Pulvertaft *et al* 1959 Irvine 1960), but subsequent investigations suggest that the cytotoxic factor is identical with the complement fixing antibody (Pulvertaft *et al* 1961, Irvine 1962)

Immune reactions of the delayed hypersensitivity type may also occur Thus Buchanan *et al* (1958) showed that intracutaneous injection of human thyroid extract in certain patients with thyroid disease gives rise to cutaneous reactions similar to those of tuberculin The importance of these immune reactions in man has not yet been clarified,

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*C. Determination of the antigen content in the thyroid gland*—If the antibodies can be bound to the antigens *in vivo*, it might be expected that the antigen content in the thyroid tissue should be lower in patients with the thyroid antibody concerned than in those without this antibody.

As the first approach to the problems has so far not been systematically utilized, this was employed in the present study.

Of the three known thyroid auto antigens, thyroglobulin is easiest to determine. Attempts were therefore made to obtain relative, but comparable measures of the thyroglobulin content in thyroid tissue, and the estimated concentrations were then related to the amount of antibody in the serum.

## MATERIALS AND METHODS

*Patients*—Thyroid tissue was studied from 71 patients who underwent operation on the thyroid gland. Of the patients studied, 39 were thyrotoxic before operation. Pre-operatively they had been treated with iodine, and 16 had for periods, also received neomercazole or methylthiouracil. A toxic adenoma was revealed in two of the toxic patients, while histological studies in the remaining 37 showed more of the other toxic tissue changes. Thirty-two goitre patients were euthyroid before

*Demonstration of thyroid auto antibodies*—The serum samples from the patients

Thyroglobulin antibody was demonstrated by Boyden's haemagglutination technique (1951) with formalin treated thyroglobulin coated sheep erythrocytes as previously described (Jørgensen & Mogenssen 1961).

while the thyroglobulin antibody gave a

strated by Donnelly's semi-micro method (1958).

*Determination of the thyroglobulin content of thyroid tissue*—At operation suitable tissue fragments were secured for histological examination and the remainder was at once stored at  $-20^{\circ}\text{C}$  until the thyroglobulin determination could be performed. Storage in this way for 1-2 months did not give rise to any change in the thyroglobulin concentrations measured. The frozen thyroid tissue was divided in blocks of a suitable size (5-10 g), weighed and cut into thin slices and coarsely homogenized with saline in a Potter homogenizer. The final volume was then adjusted to 10 ml per gramme of tissue. The extracts were allowed to stand overnight at  $4^{\circ}\text{C}$ , after which the sediment was removed by centrifugation. In the majority of cases two extracts were made from each gland, usually one from each



but experiments with immunization of guinea pigs with homologous thyroglobulin have revealed a correlation between the occurrence of experimental thyroiditis and positive delayed skin thyroglobulin reaction, whereas no relationship was demonstrated between the development of thyroiditis in the animals and the presence of thyroglobulin antibody (Miescher *et al* 1961, McMaster *et al* 1961). Felix-Davies & Waksman (1961) were even able to transfer experimental thyroiditis in guinea pigs by means of lymphocytes. Simultaneously with the development of thyroiditis in the recipients, a positive cutaneous reaction to thyroglobulin occurred, whereas it was not possible to demonstrate any thyroglobulin antibody in the animals.

When, for the first time, Roitt *et al* (1956) demonstrated a precipitating thyroid auto-antibody (thyroglobulin antibody) in the serum of patients with Hashimoto's disease, it was reasonable to assume that a causal relationship existed between the auto-immune reaction and the progressive destruction of the thyroid tissue which is characteristic of this disease. Definite evidence in support of this assumption was produced by Witebsky *et al* who demonstrated that active immunization of rabbits with autologous thyroid extract (to which Freund's adjuvant was added) simultaneously with an auto-antibody production led to histological changes in the remaining thyroid tissue in the rabbits—changes, which were of essentially the same type as those seen in Hashimoto's disease (Witebsky & Rose 1956, Rose & Witebsky 1956). However, when it was realized that a number of different forms of thyroid auto-immune reactions exist, and that the auto-immune reactions may occur not only in Hashimoto's disease, but also in other thyroid disorders and even in persons without signs of such disease (Hill 1961), the problems involved have become fairly complicated. At the present time, attempts must be made, as far as possible, to assess the importance of each of the various forms of thyroid auto-immunization separately. In man, such an assessment may be performed on the following principles:

*A Clinical evaluation*—Clinical observations have given reasons to believe that severe cases of auto-immunization will often end in myxoedema. This has been confirmed by several recent investigations, which have concordantly shown that postoperative myxoedema mainly occurs among patients with complement-fixing antibody in the serum at the time of operation (Gammie *et al* 1961, Buchanan *et al* 1962, Hjort & Mogensen 1962, Irvine *et al* 1962).

*B Transfer of thyroid antibodies*—Nature herself performs this experiment by the passage of the antibodies through the placenta from the maternal to the foetal circulation. Thus, large amounts of passively transferred thyroglobulin antibody and complement-fixing antibody have been demonstrated in newborn infants, apparently without having exerted any influence on thyroid function (Blizzard *et al* 1960, Parker & Beurvalles 1961, Hjort & Pedersen 1962).

*C Determination of the antigen content in the thyroid gland*—If the antibodies c might be expected that the ant be lower in pa tients with tiose without this antib

As ally

Of the three known thyroid auto-antigens, thyroglobulin is the most important. Attempts were therefore made to obtain relative, but comparable measures of the thyroglobulin content in thyroid tissue, and the estimated concentrations were then related to the amount of antibody in the serum

## MATERIALS AND METHODS

*Patients*—Thyroid tissue was studied from 71 patients who underwent operation on the thyroid gland. Of the patients studied, 39 were thyrotoxic before operation, 13 had, for periods, also toxic adenoma, and 19 were euthyroid. Of the 39 thyrotoxic patients, 19 had toxic adenoma, and 20 had diffuse toxic goitre. Of the 13 patients with toxic adenoma, 7 had toxic goitre, and 6 had toxic adenoma. Of the 19 euthyroid patients, 10 had toxic goitre, and 9 had toxic adenoma. All patients were euthyroid before operation.

*Antibody against thyroglobulin*—The serum samples from the patients

previously described (Hjort & Mogensen 1961)

Antibody against second colloid antigen (CA 2 antibody) was demonstrated by Coombs' fluorescent test (sandwich technique) performed on methanol fixed frozen sections of thyroid tissue (approx 6  $\mu$ ) as described by Balfour *et al* (1961). Com-

Positive serum of medium strength was included as a control. As thyroglobulin antibody also stains the colloid, only sera which had yielded a negative or very weakly positive reaction by the haemagglutination test were studied. In the presence of CA 2 antibody, uniform bright apple green staining of the colloid was observed—as described by Balfour *et al* (1961)—while the thyroglobulin antibody gave a floccular appearance of the colloid space.

Complement fixing antibody was demonstrated by Donnelley's semi-micro method (1931) as described by Rott & Danneberg (1958).

*Determination of the thyroglobulin content of thyroid tissue*—At operation, suitable tissue fragments were secured for histological examination, and the remainder was at once stored at  $-20^{\circ}\text{C}$  until the thyroglobulin determination could be performed. Storage in this way for 1–2 months did not give rise to any change in the thyroglobulin concentrations measured. The frozen thyroid tissue was divided in blocks of a suitable size (5–10 g), weighed and cut into thin slices and coarsely homogenized with saline in a Potter homogenizer. The final volume was then adjusted to 10 ml per gramme of tissue. The extracts were allowed to stand overnight at  $4^{\circ}\text{C}$ , after which the sediment was removed by centrifugation. In the majority of cases, two extracts were made from each gland, usually one from each

TABLE 1  
*Immunological Determination of the Thyroglobulin Content in Thyroid Tissue.*

Extract dilution	Dilution of test serum containing thyroglobulin antibody										Thyroglobulin concentration ( $\mu\text{g/ml}$ )	1 extracted thyroglobulin (mg g tissue)
	1	100	1	500	1	2000	1	4000	1	8000	1	16000
Extract 195	1 10 000	+	+	0	0	0	0	0	0	0	0	100
	1 20 000	+	+	2	0	0	0	0	0	0	0	100
	1 40 000	+	+	+	1	0	0	0	0	0	0	100
	1 80 000	+	+	+	+	0	0	0	0	0	0	>80
	1 160 000	+	+	+	+	+	0	0	0	0	0	>80
Extract 196	1 10 000	+	+	2	0	0	0	0	0	0	0	50
	1 20 000	+	+	+	1	0	0	0	0	0	0	>40
	1 40 000	+	+	+	+	1	0	0	0	0	0	1
	1 80 000	+	+	+	+	+	2	0	0	0	0	0.5
	1 160 000	+	+	+	+	+	+	0	0	0	0	>0.2
Thyroglobulin Standard	20 $\mu\text{g/ml}$	2	2	0	0	0	0	0	0	0	0	40 to 50
	10	+	+	0	0	0	0	0	0	0	0	40
	5	+	+	2	0	0	0	0	0	0	0	40
	2	+	+	+	2	0	0	0	0	0	0	40
	1	+	+	+	+	2	0	0	0	0	0	40
	0.5	+	+	+	+	+	2	0	0	0	0	40
	0.2	+	+	+	+	+	+	1	0	0	0	40
	0.1	+	+	+	+	+	+	+	1	0	0	40
	0	+	+	+	+	+	+	+	+	0	0	40
	0	+	+	+	+	+	+	+	+	+	0	40

4 = complete agglutination  
 3 = large ring  
 2 = medium-sized ring  
 1 = small ring  
 0 = no agglutination

lobe but in a few casts the amount of tissue was so small that only one extract

groups in the extracts  
previously used for the  
om the stock dilution of

tion in  
dilution

formalin treated, thyroglobulin coated erythrocytes was used as a standard. It was left overnight at room temperature and read next morning. It appeared to be appropriate to distinguish between various grades of agglutination. 4 was used for complete agglutination (i.e. sedimentation like a carpet), 3 for a large ring, 2 for a medium sized ring, 1 for a small ring and 0 for a negative reaction.

A comparison of the haemagglutination inhibitions produced in the various

extract  
globulin  
globulin  
content  
of tissue  
compar

as this. However, as the same standard and the same thyroglobulin anti body-containing test serum were used in all the determinations the values will be comparable which was the only object in view.

TABLE 2

*The Influence of the Reaction Time on Immunological Determination of Thyroglobulin*

Extract	Reaction time (in hours) at 2 ° C				
	1 hour	2 hours	4 hours	8 hours	24 hours
1	180	180	180	180	180
2	80	60	80	80	80
3	40	> 40	60	60	60
4	60	160	180	180	180
5	20-40	40	40	40	40

The figures indicate the amounts of thyroglobulin in mg/g tissue

Although the procedure is very similar to that used for the determination of thyroglobulin in serum the influence of certain factors must nevertheless be considered in some detail.

before the thyroglobulin coated erythrocytes were added. The results (Table 2) showed that the antigen antibody reaction seemed to have been completed at 4 hours in all cases. As early as at 1 hour the standard exhibited the very constant inhibition pattern, as appears from Table 1).

2 *Influence of dilution on the extraction of thyroglobulin*—In two cases homogenized saline extracts were prepared in the dilutions 1:2, 1:10, 1:20 and 1:40. As the thyroglobulin determinations gave the same results on all extracts from the same gland the dilution normally used i.e. 1:10 must with great certainty result in a fairly complete extraction of the thyroglobulin.

3 *Destruction of thyroglobulin in the extracts*—It is conceivable that when the extracts are allowed to stand overnight at 4° C, this might result in a certain decomposition of the thyroglobulin caused by co-extracted enzymes. In order to throw light on this problem, a large amount of extract was prepared in a cooled mixer (Ato Mix) from each of two goitres. By this procedure which requires relatively large quantities of thyroid tissue complete homogenization is obtained. Each of the two extracts were distributed in six tubes. One sample of each extract was centrifuged at once and the supernatant stored at -20° C. The remaining tubes were left at 4° C. On each of the next five days one sample of each extract was centrifuged and the supernatant stored at -20° C. Simultaneous thyroglobulin determinations were then performed on all the samples. The results (Table 3) show that no destruction of the thyroglobulin in the extracts could be disclosed after standing at 4° C.

TABLE 3

*The Results of Immunological Determination of Thyroglobulin after Standing of the Extracts at 4° C for Various Periods*

Time of standing (in 24 hour periods) at 4° C					
0	1	2	3	4	5
160	160	160	180	160	180
200	200	200	200	200	200

The figures indicate the amounts of thyroglobulin in mg/g tissue

TABLE 4

*Thyroglobulin Content in Different Extracts from each of Three Goitres*

Type of goitre		
Non-toxic nodular	Toxic	Toxic
100-125	110	150-200
100-125	110	200
125	110	200
125	100-125	200
125	125	200
	125	200-250
	125	
	125	
	125-150	
	130	
	160	

The figures indicate the amounts of thyroglobulin in mg/g tissue

4 *Uniformity of the thyroid tissue*—In order to assess to what extent the result obtained in the study of 10 g tissue can be taken as representative of the thyroid gland concerned a series of extracts from different parts of the same goitre were studied in three cases. The results are shown in Table 4. As the technique used in the present study merely aims at obtaining comparable levels of the thyroglobulin

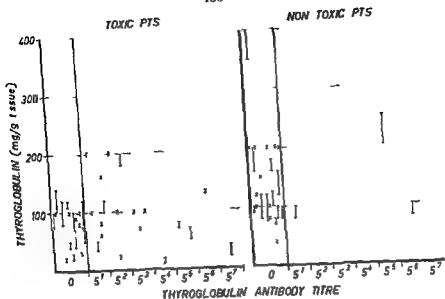


Fig 1

Thyroglobulin concentrations in thyroid tissue related to the amounts of thyroglobulin antibody in the serum

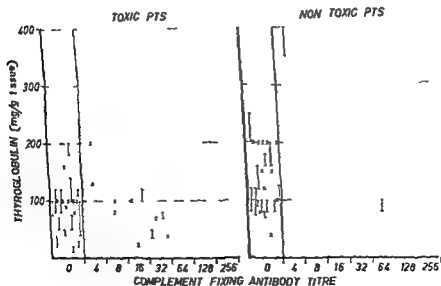


Fig 2

Thyroglobulin concentrations in thyroid tissue related to the amounts of complement fixing antibody in the serum

concentrations in different glands, the agreement between the various results obtained in all three cases must be regarded as fully satisfactory. It also appears from Figs. 1 and 2 that fairly good agreement between the results for the two different extracts which were routinely prepared from each gland was generally obtained.

In electrophoretic analysis, thyroglobulin migrates as an  $\alpha$  globulin. Thyroglobulin will generally constitute the greater part of the  $\alpha$ -globulin in the extracts, and at any rate, the concentration of thyroglobulin in the tissue will not exceed that of  $\alpha$  globulin. Attempts were therefore made to obtain a control of the immunological thyroglobulin determinations by estimating the amount of  $\alpha$  globulin in some of the extracts. The protein concentrations in the extracts were determined by a biuret reaction. Paper electrophoresis was performed with an LKB apparatus according to the hints suggested by the manufacturer, i.e. Schleicher & Schuell 2043 B paper and a barbital buffer (ionic strength 0.125, pH 8.6) were employed. The electrophoresis was run for about 18 hours with a potential gradient of 2 V/cm. The strips were stained with bromophenol blue and read in an Ithor photometer.

TABLE 5  
Antibodies Found in the 71 Patients Studied

Histological diagnosis	No of patients	TGA + CFA	CA 2 + CA 4	IGA	CA 2	CA 4	No anti bodies
Diffuse toxic changes	37	8	0	12	4	3	9
Toxic adenoma	2	0	0	1	0	0	1
Non toxic colloid goitre (diffuse or nodular)	27	1	1	2	2	0	21
Non toxic adenoma	5	1	0	0	0	0	4

TGA thyroglobulin antibody      CA 2-A, CA 4 antibody  
CFA complement-fixing antibody

## RESULTS

The thyroid auto-antibodies revealed in the sera from the 71 patients are shown in Table 5. In nearly all the patients who had thyroid antibodies in the serum (including most of those with CA-2-antibody only), the histological examination revealed lymphocytic infiltration in the thyroid tissue. The presence of lymphocytic infiltration may, by displacement of the glandular tissue, give rise to misinterpretation of the thyroglobulin concentration in the active thyroid tissue. Only in two patients, viz. the only one with non-toxic goitre who had both thyroglobulin antibody and complement-fixing antibody in the serum and the toxic patient who had the highest thyroglobulin-antibody titre, were the lymphocytic infiltrations in the thyroid tissue so pronounced that the actual glandular tissue had been displaced. In the other cases with lymphocytic infiltration, the infiltrates constituted such a small fraction of the tissue that this cannot have exerted any appreciable influence on the results obtained.

In Fig. 1 the thyroglobulin levels revealed in the 37 toxic and 27 non-toxic glands with diffuse changes are related to the amounts of thyroglobulin antibody in the serum. It appears that the values for both groups are scattered over a wide range. On the whole, the thyroglobulin content was higher in the non-toxic than in the toxic glands. In another connection, Wilebsky *et al.* (1957) made similar observations. On the basis of the present investigation it cannot be said if the lower average level in toxic patients is solely referable to the disease, or if the pre-

operative treatment also plays a part. The results in Fig 1 do not suggest any immediate relationship between the amounts of free, thyroglobulin antibody-fixing groups in the thyroid gland and the occurrence of thyroglobulin antibody in the serum. There are thus no signs suggesting that the circulating thyroglobulin antibody *in vivo* is capable of neutralizing the antigen in the colloid follicles in the thyroid gland.

In Fig 2, the thyroglobulin concentrations in the glandular tissue are related to the amounts of complement fixing antibody in the serum. Among the thyrotoxic patients who had complement-fixing antibody it seems as if the thyroglobulin concentrations decreased with increasing antibody titres, but taken collectively, the results obtained in the patients with antibody do not differ from the observations made in those without antibody.

In two of the eight patients in whom CA-2 antibody was demonstrable in the glandular tissue, the concentration of this antibody was high in the remaining six patients.

In the seven cases in which the determinations were performed on tissue from solitary adenomata, relatively low thyroglobulin concentrations were observed. The results are shown in Table 6.

TABLE 6  
*Thyroglobulin Concentrations in the Seven Adenomata Studied*

Histological diagnosis	Antibody titre		Thyroglobulin concentration (mg/g tissue)
	TGA	CFA	
Toxic adenoma	25	0	20
do	0	0	50
Non toxic adenoma	625	4	35-70
do	0	0	80-100
do	0	0	10-15
do	0	0	25
do	■	■	80

In Fig 3, the concentrations of  $\alpha$ -globulin in the thyroid tissue—here taken as an expression of the maximum amount of thyroglobulin which may occur in the tissue—are related to the amounts of thyroglobulin antibody in the serum. The dispersion is not as great as for the thyroglobulin concentrations (Fig 1), and the values obtained are of the same order as the thyroglobulin concentrations which DeGroot & Carvalho (1960) revealed in experiments with ultracentrifugation. However, Fig 3 does not either reveal any relationship between the  $\alpha$ -globulin concentrations in the thyroid tissue and the thyroglobulin-antibody titre in the serum.

In some of the cases, good agreement between the concentrations of thyroglobulin and  $\alpha$ -globulin was observed. However, this scarcely



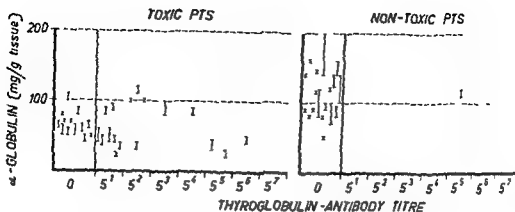


Fig 3

$\alpha$ -Globulin concentrations in thyroid tissue related to the amounts of thyroglobulin antibody in the serum

means that the  $\alpha$ -globulin fractions and the thyroglobulin standard used have consisted of pure thyroglobulin, but it should rather be taken as indicating that the  $\alpha$ -peak has here been of the same composition as the purified thyroglobulin fraction which was used as the standard (in the electrophoretic analysis, it migrated as an almost pure  $\alpha$ -peak). However, in other cases, the two values showed considerable divergence. In order to throw light on this discrepancy, the ratio of the thyroglobulin concentration (in cases in which this could only be fixed at an approximate level, a mean value was used) to the concentration of  $\alpha$ -globulin was calculated 
$$I = \frac{\text{concentration of thyroglobulin in thyroid tissue}}{\text{concentration of } \alpha\text{-globulin in thyroid tissue}}$$

These values will obviously be beset with a considerable uncertainty, which also appears from the fact that in some of the cases the two results which were obtained independently of each other from the same gland showed a fairly wide difference. From Fig 4, in which the calculated ratio is related to the amount of thyroglobulin antibody in the serum, it is seen that considerable variations occur from patient to patient. If, *in vivo*, the thyroglobulin antibody is bound to the thyroglobulin in the thyroid gland, it might be expected—even if the phenomenon may be equalized through an increased production of thyroglobulin—that the ratio was lower in patients with thyroglobulin antibody than in those without this antibody. However, such a tendency is not seen in Fig 4. On the contrary, some of the highest values were found among the patients with thyroglobulin antibody, and only a few of these patients had a ratio of approx 1. C1-2-antibody was present in two of the three patients who seemed to form a separate group with exceptionally low ratios (below 0.6).

Thus, the results in Fig 4 might suggest that the thyroglobulin extracts from some of the patients, including most of those with thyroglobulin auto-immunization, have a relatively greater thyroglobulin-antibody-fixing capacity (in relation to the amount of  $\alpha$ -globulin) than

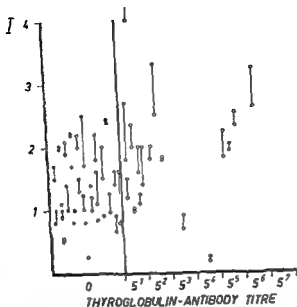


Fig 4

The ratio between the concentrations of thyroglobulin and  $\alpha$  globulin in thyroid tissue ( $I$ ) related to the amounts of thyroglobulin antibody in the serum

○ Toxic patients

● Non toxic patients

the extracts from the majority of the patients without thyroglobulin antibody. This might be due either to the presence of antibody-fixing groups outside the  $\alpha$  globulin fraction or to a changed composition of the  $\alpha$  globulin fraction in the extracts, including a changed composition of the thyroglobulin itself.

In an attempt to throw light on the last mentioned possibility a purified thyroglobulin fraction was prepared by the method of *Derrien et al* (1948) from each of 11 goitres originating from patients with or without thyroglobulin antibody. After dialysis and freeze-drying, solutions containing from 2 to 20  $\mu\text{g}$  of the thyroglobulin fractions per ml were prepared, and amounts of 0.1 ml of these solutions were mixed with 0.1 ml of diluted test serum (1:100) containing thyroglobulin antibody. After standing at  $37^\circ\text{C}$  for 4 hours, thyroglobulin coated erythrocytes were added. The resultant pattern of haemagglutination inhibition is shown in Table 7, in which the thyroglobulin antibody titre in the serum and the ratio ( $I$ ) between the amounts of thyroglobulin and  $\alpha$  globulin in the extracts studied are also listed. These purified thyroglobulin fractions, which in electrophoretic analysis migrated as almost pure  $\alpha$  peaks, did not show quite as great variations in their thyroglobulin antibody-fixing capacity as seen in Fig 4. However, in repeated determinations, the fractions from the four patients with the lowest antibody titres (Nos 7-10) differed unquestionably from the standard and from the fractions which originated from patients without thyro-

globulin auto immunisation. In No. 8 (Table 7), which in spite of the presence of thyroglobulin antibody revealed almost equally large amounts of thyroglobulin and  $\alpha$ -globulin in the two extracts studied, the thyroglobulin fraction thus fixed about 50 per cent more antibody than the standard fraction. On the other hand, the two cases with higher antibody titres (Nos. 11 and 12, Table 7) did not differ from the standard. The experiment thus lends support to the view that variations may occur in the composition of the  $\alpha$ -globulin fraction of the thyroid extracts, but the antibody-fixing capacity of the thyroglobulin fractions scarcely vary so much that it will be permissible to draw any conclusions as to a change in the composition of the thyroglobulin molecule itself.

TABLE 7

*The Antibody Fixing Effect of the Thyroglobulin Fractions from Various Glands*

No.	Fluorescence	t	Amount of thyroglobulin fraction ( $\mu\text{g} \approx 1 \text{ ml}$ ) to which 0.1 ml test serum was added (dil. 1:100)									
			0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0
1	0	Standard	4	4	4	4	4	4	4	0	0	0
			4	4	4	4	4	4	4	1	0	0
2	0*	0.25	4	4	4	4	4	4	4	4	4	3
			4	4	4	4	4	4	4	4	4	3
3	0	0.8-1.0	4	4	4	4	4	4	2	0	0	0
			4	4	4	4	4	4	3	0	0	0
4	0	0.8	4	4	4	4	4	4	4	0	0	0
			4	4	4	4	4	4	4	0	0	0
5	0	1.1	4	4	4	4	4	4	2	0	0	0
			4	4	4	4	4	4	0	0	0	0
6	0*	2.2-2.2	4	4	4	4	4	4	4	0	0	0
			4	4	4	4	4	4	4	0	0	0
7	5	4.0-4.5	4	4	4	4	4	1	0	0	0	0
			4	4	4	4	4	0	0	0	0	0
8	5	1.1-1.25	4	4	4	4	4	0	0	0	0	0
			4	4	4	4	2	0	0	0	0	0
9	25	2.0-1.8	4	4	4	4	4	2	0	0	0	0
			4	4	4	4	4	2	0	0	0	0
10	125	0.7-0.9	4	4	4	4	4	2	0	0	0	0
			4	4	4	4	4	2	0	0	0	0
11	625	3.5-1.2	4	4	4	4	4	4	2	0	0	0
			4	4	4	4	4	3	1	0	0	0
12	3125	1.8-2.2	4	4	4	4	4	4	3	0	0	0
			4	4	4	4	4	4	2	0	0	0

The figures indicate the degrees of haemagglutination. See Table 1.  
\* Serum contained CA 2 antibody.

## DISCUSSION

The studies presented here did not suggest that the thyroglobulin antibody *in vivo* is capable of neutralizing the antigen in the thyroid gland. This result does not agree with the observation made by Rose &

Witebsky (1956) after active immunization of rabbits by means of thyroid extract to which Freund's adjuvant was added. In their experiments the amount of antigen was the same, but the amount of circulating antibody and the decrease in the antigen content was observed. The discrepancy may perhaps be explained by the active immunization of the rabbits by the use of Freund's adjuvant (with development of delayed hypersensitivity?).

That it was necessary here to employ the rather troublesome round-about method of comparing the amount of free, thyroglobulin-antibody-fixing groups in the thyroid tissue with that of  $\alpha$  globulins, is due to the fact that it was not possible to produce an absolutely pure thyroglobulin (Shulman & Witebsky 1960), and just this fact renders the evaluation of the results difficult. As regards the relation between the amounts of thyroglobulin-antibody-fixing groups and  $\alpha$  globulin, the composition of the extracts seems to vary within fairly wide limits. The results obtained are compatible with the view that the relative content of thyroglobulin groups seems to be able to increase from a certain basic level, which is found mainly in patients without thyroglobulin auto-immunization, to a higher level which occurs in some patients without signs of production of antibody against thyroglobulin, but especially in patients in whom thyroglobulin antibody is present. On the other hand, a level which was appreciably lower than the basic one was observed in three patients, including two who had CA 2 antibody in the serum. (The relatively low thyroglobulin content might perhaps be due to a relatively higher content of second colloid antigen.)

It is a well known fact that the complement-fixing antigen is present in the greatest amounts in glandular tissue from thyrotoxic patients (Roitt & Doniach 1960), and apparently also occurs in a high concentration in the active glandular tissue in Hashimoto's disease (Pulverfitt *et al* 1961). Thus, the greatest amounts of antigen are seen in the groups of patients in whom the occurrence of the corresponding complement fixing antibody is most frequent. Accordingly, it would seem reasonable to assume that patients with production of antibody against thyroglobulin must be sought among those with a relatively high thyroglobulin antibody-fixing (antigenic?) activity in the glandular tissue. A higher antigen level might be either the result of the immunization processes (an overcompensation phenomenon?) or the cause of the development of auto-immunization. The fact that the complement fixing antigen may be present in a high concentration in the tissue without being accompanied by complement fixing antibody in the serum, and that a high thyroglobulin activity may occur in the absence of thyroglobulin antibody, weighs against the former possibility.

Both as far as the complement-fixing antigen and thyroglobulin are concerned, it applies that the higher antigen level might be the result either of purely quantitative changes in the composition of the proteins

of the thyroid gland or of qualitative changes, so that the individual antigen molecule might contain more antibody-fixing (antigenic?) groups than usual. The latter possibility would explain the development of auto-immunization, *i.e.* the disease would be the primary and the auto-immune reaction a secondary phenomenon caused by changes in the antigen structure. These considerations are based on studies on the conditions in patients with thyrotoxicosis or non-toxic goitre, and the assumptions do therefore not exclude the view that the auto-immune reactions in other thyroid disorders—lymphadenoid goitre, primary myxoedema—might be of a primary pathogenic significance and be directed against normal antigen molecules. If absolutely pure antigen could be produced, it would be easy to decide if both possibilities might actually occur.

### SUMMARY

In 71 patients who underwent operations on the thyroid gland, attempts were made to obtain relative, but comparable measures for the thyroglobulin concentrations in the thyroid tissue by means of an immunological method, *viz.* a haemagglutination-inhibition technique, which is described. At the same time, the patients were studied for thyroglobulin antibody, complement-fixing thyroid antibody and CA 2-antibody. No correlation between the thyroglobulin concentrations in the thyroid tissue and the occurrence of thyroid auto-antibodies in the serum could be demonstrated.

As thyroglobulin in electrophoresis migrates as  $\alpha$ -globulin, the content of  $\alpha$ -globulins in the thyroid tissue was determined in a number of the cases in order to obtain a measure of the maximum amount of thyroglobulin which might occur in the tissue. In some cases, good agreement was found between the amounts of thyroglobulin determined by the immunological method and the content of  $\alpha$ -globulin, while other cases—especially patients in whom thyroglobulin antibody was demonstrated in the serum—showed a discrepancy between these two values, the thyroglobulin concentrations relatively being too high.

As the purified thyroglobulin fractions also showed some variations in their thyroglobulin antibody fixing capacity, it is discussed if the exciting factor in thyroglobulin auto-immunization may be a change in the composition of the thyroglobulin depot of the thyroid gland, or if a change in the thyroglobulin molecule may occur so that it contains more antigenic groups than usual.

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## TISSUE REACTION TO BARIUM SULPHATE CONTRAST MEDIUM

By

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Received 1953

Over a number of years we have now and then noticed peculiar granulomatous lesions in the abdomen. Correlation of clinical and pathological data in such cases suggested that the granulomatous changes were caused by barium sulphate which after X ray investigation had leaked through a peptic ulcer either in the gastric or duodenal wall into the peritoneal cavity. In all the cases studied the granulomatous changes were chiefly located in the greater omentum. As the lesion differs from barium granulomas previously reported in the literature we shall describe the principal features of one of our cases where the phagocytizing cells in the granulomas have been investigated and analyzed with the aid of light microscopy, contact microradiography and X ray microfluorescence.

The tissue block investigated was obtained from a man 58 year old who had suffered from gastric pains for about 10 years. After "back operation" we performed a total gastrectomy. At the operation ad modum Billroth + enterogastrostomy two thirds of the gastric wall and part of the duodenum was removed.

### LIGHT MICROSCOPY

Microscopically a peptic ulcer was evident in the duodenal wall. In the connective tissue around the duodenum and in the omental fat could be seen areas with large cells well filled with cytoplasm containing small nuclei and dense chromatin (Fig. 1). On staining with haematoxylin-eosin the cytoplasm of the cells was greyish and showed fine granules which clearly refracted the light.

No macrophages or plasma cells. No barium crystals were seen microscopically, nor had contrast medium been ex-



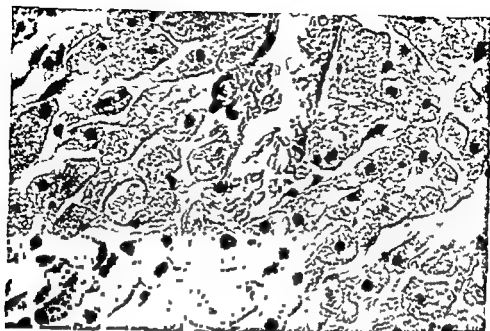


Fig. 1

Cell containing cytoplasmatic barium granules seen in transmitted light from the subserosal connective tissue in the gastric wall

sible to the naked eye. *PAD: Ulcus pepticum chron perforatum (?) duodeni, bariumgranulomas*

#### CONTACT MICORADIOGRAPHY

I. Sections  $7\mu$  in thickness from the paraffin embedded tissue were placed in close contact with a film emulsion (Kodak Spectroscopic Plate No. 649). They were then exposed to X rays for three minutes at 10 KV and 10 mA (Machlett X ray tube OLG 50A, Cu mode, 1.5 mm focal spot, 1 mm beryllium filter). The cells described in the previous section appeared on the film to have white cytoplasm, due to the high X ray absorption of this part of the cell, in contrast to the general tissue background. The nucleus did not absorb any X ray and appeared as a black spot in the cytoplasm when exposed to X rays within the wave length region used, 1.2-4 Å (Fig. 2).

II. Similar sections, with a thickness of  $5\mu$ , cut from the same tissue block were exposed to X rays within the wave length region 8-10 Å using the same technique (Cu anode, 35 KV, 35 mA, exposure time 15 minutes). The micro radiograms obtained show the mass distribution within the section, according to Engstrom & Lindstrom (1950). The microradiograms could also be used for location of the highly X ray absorbing cells in the organic stroma within the section (Fig. 3).

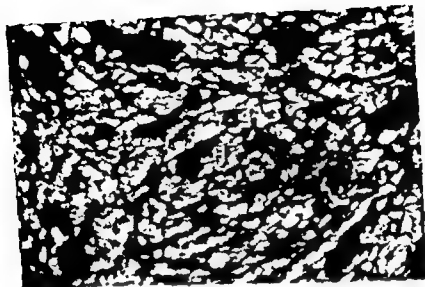


Fig 2

Microdiagram obtained in the soft X ray region (see text) from cells containing barium sulphate from the same area as in fig 1

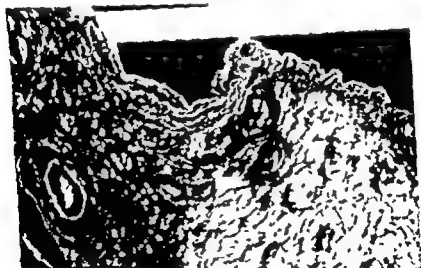


Fig 3

#### X RAY FLUORESCENCE MICRO ANALYSIS

About five isolated cells (dissected out by hand under a binocular microscope) were bombarded with X-rays from an X-ray microscope (type Cosslett Nixon, Cu mode, 15 kV, 150 nA). The cells were placed



Fig. 1.

Cell containing cytoplasmatic barium granules seen in transmitted light from the subserosal connective tissue in the gastric wall

sible to the naked eye *P A D : Ulcus pepticum chron. perforatum (?) duodeni, bariumgranulomas*

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II Similar sections, with a thickness of  $5\ \mu$ , cut from the same tissue block were exposed to X-rays within the wave length region 8–10 Å, using the same technique (Cu anode, 35 KV, 35 mA, exposure time 15 minutes) The micro-radiograms obtained show the mass distribution within the section, according to Engstrom & Lindstrom (1950) The microradiograms could also be used for location of the highly X-ray absorbing cells in the organic stroma within the section (Fig. 3)

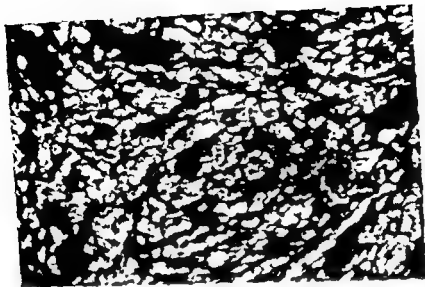


Fig 2

Microradiogram obtained in the soft X ray region (see text) from cells containing barium sulphate. From the same area as in Fig 1



Fig 3

Microradiogram obtained in the ultrasoft X ray region showing the mass distribution within the section. The area with barium containing cells is seen to the right

#### X RAY FLUORESCENCE MICRO ANALYSES

About five isolated cells (dissected out by hand under a binocular microscope) were bombarded with X rays from an X ray microscope (type Cowell-Nixon, Cu mode, 10 kV, 100 nA). The cells were placed



Fig. 1.

Cell containing cytoplasmic barium granules seen in transmitted light from the subserosal connective tissue in the gastric wall

sible to the naked eye *P A D · Ulcus pepticum chron perforatum (?) duodeni, bariumgranulomas*

### CONTACT MICORADIOGRAPHY

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berger 1928, Bittrolff 1928, Himmelmann 1932, Singer 1934, Strasser 1946, Kay 1954). Most of these cases have had peptic ulcers in the gastric or duodenal wall which have perforated either before or during the X ray investigation. In a minor number of cases leakage of contrast medium has occurred through the appendix, colic, sigmoid or rectal walls during the administration of barium enemata.

The tissue reactions caused by the leakage of barium sulphate from the digestive tract have been studied by a few authors (Kay 1954 and Mendeloff 1956). The effect of endotracheal and intraperitoneal injection of barium sulphate suspensions has been studied under experimental conditions by Kleinsasser & Warshaw (1952) and Huston, Wallack & Cunningham (1952).

The barium granulomas seen in our material differ somewhat from those described by the latter authors. The granuloma (Mixobar) is composed of very fine particles and that the small grains are probably all caught by cell phagocytosis.

In our experience barium granulomas may sometimes cause the pathologist diagnostic difficulty. In one of our cases the greyish white areas were considered by the surgeon as false granulomas following a previous operation on the abdomen. Sometimes the clinical data available to the pathologist are not of the kind which might give him an association leading to the correct diagnosis.

In order to get an idea of how long the barium granulomas might be detectable after their development white rats were given 0.8 cm<sup>2</sup> barium sulphate (Mixobar) intraperitoneally. A week after the injection granulomatous spots were seen in the fat tissue of the greater omentum and in the peritoneum. Microscopically the spots were all like barium granulomas in man. One rat was allowed to live seven months after the injection and small greyish white plaques were seen in the peritoneal cavity. The refringence and the granules of the cells in this instance were less distinct than those from fresh granulomas and the microscopic picture showed fewer granules in the cytoplasm.

## SUMMARY

Details of a case of barium granuloma in the peritoneal cavity, caused by leakage of barium contrast medium (Mixobar) through a peptic ulcer in the duodenum are described. The granuloma showed mainly large cells containing cytoplasm filled with small refringent granules. The cells were investigated by contact microradiography, and X ray fluorescence micro-analysis and proved to contain barium sulphate. Comparison of the X ray spectrum of the intracellular granules and a sample of barium contrast medium used for clinical diagnostic purposes proved to be identical.

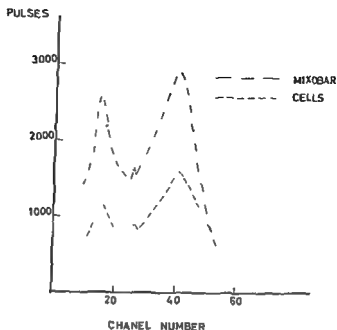


Fig 4

X-ray fluorescence curves as seen on the oscilloscope. Top curve from the contrast medium, bottom curve from the cells

on top of an electron microscope aperture with a diameter of  $200\ \mu$  in an evacuated chamber. Within the specimen secondary X-rays were generated, i.e. X-ray fluorescence radiation, whose spectral definition is due to the atomic composition of the specimen. The intensities at different wavelengths of the fluorescence radiation were analyzed with the aid of a proportional counter and a pulse height multichannel analyzer and made visible on an oscilloscope. A curve was obtained and along the x-axis different energies of wavelengths were plotted. Along the ordinate the height of the peaks of the curve represented the intensity at a particular wavelength, i.e. the amount of an element. Each element has been shown to have a separate peak (Long & Rockerl 1963).

Using this method the cells analyzed produced peaks at wavelengths corresponding to the emission peaks of barium (Fig 4). A comparative investigation made with a small drop of Mixobar, the same contrast medium used for the clinical gastric X-ray examination, produced peaks identical with those from the cells. The only difference was the height of the peaks due of course to the larger amount of material analyzed. This confirmed that the cytoplasm of the cells analyzed contained barium contrast medium.

#### DISCUSSION

Leakage of barium contrast medium into the abdominal cavity during gastric X-ray examination has been shown in number of cases (Am-

berger 1928, Bittroff 1928, Himmelmann 1932, Singer 1934, Strasser 1946, Kay 1954) Most of these cases have had peptic ulcers in the gastric or duodenal wall which have perforated either before or during the X ray investigation. In a minor number of cases leakage of contrast medium has occurred through the appendix, colic, sigmoid or rectal walls during the administration of barium enema.

The tissue reactions caused by the leakage of barium sulphate from the digestive tract have been studied by a few authors (Kay 1954 and Mendeloff 1956). The effect of endotracheal and intraperitoneal injection of barium sulphate suspensions has been studied under experimental conditions by Kleinsasser & Warshaw (1952) and Huston, Wallick & Cunningham (1952).

The barium granulomas seen in our material differ somewhat from those described in the literature. We have not seen the extracellular particles described by Kay and Mendeloff, nor the giant cells described by the latter. The reason for this may be that the barium sulphate used (Mixobar) is composed of very fine particles and that the small grains are probably all caught by cell phagocytosis.

In our experience barium granulomas may sometimes cause the pathologist diagnostic difficulty. In one of our cases the greyish white areas were considered by the surgeon as talc granulomas following a previous operation on the abdomen. Sometimes the clinical data available to the pathologist are not of the kind which might give him an association leading to the correct diagnosis.

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## SUMMARY

Details of a case of barium granuloma in the peritoneal cavity, caused by leakage of barium contrast medium (Mixobar) through a peptic ulcer in the duodenum, are described. The granulomas consist of only large cells containing many small granules. The granules fluoresce under short wave light. Comparison of the fluorescence spectrum of the intracellular granules and a sample of barium contrast medium used for clinical diagnostic purposes proved to be identical.



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## ADENOSINE TRIPHOSPHATASE ACTIVITY OF RAT SKIN IN EARLY WOUND HEALING

By

J. RAEEKALLIO and Eeva LEVONEN

Received 24.1.63

In previous investigations the early appearance of some hydrolytic and oxidative enzymes (Raekallio 1960, 1961) and of transglutosylases (Raekallio & Levenon 1962) has been demonstrated in healing wounds. Among the specific phosphatases adenosine triphosphatase (ATPase) plays an essential rôle in various metabolic processes. In this investigation, therefore, its appearance and distribution in the skin has been studied during the first 24 hours of wound healing.

### MATERIAL AND METHODS

Male rats of the Wistar-Kyoto strain, weighing 150-200 g, were used. The rats were anesthetized with ether and a skin flap containing the wound was removed immediately and frozen with solid carbon dioxide for sectioning in a cryostat.

Sections were cut at 15  $\mu$ , fixed for 10 minutes in ice cold neutral formal calcium (Vosik ff et al 1961) washed in one per cent calcium chloride solution for two minutes, incubated for 15 minutes in a substrate solution of Padykula & Herman (1955) and further processed according to their method. For control sections a glycer phosphate was used instead of ATP in the substrate solution.

### RESULTS

Brown black deposits of lead sulphide indicated ATPase activity in the sections. In the uninjured skin farther away from the wound moderate to intense ATPase activity was observed in occasional fibroblasts. Most of them were situated in the papillary layer of the dermis, especially in the vicinity of the dermoepidermal junction where the capillaries also showed intense activity. The panniculus carnosus and the striated muscle were intensely or moderately stained. Ac-

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## ADENOSINE TRIPHOSPHATASE ACTIVITY OF RAT SKIN IN EARLY WOUND HEALING

19

J. RAEBALLIO and F. A. LEVONEN

Received 24.1.83

In previous investigations the early appearance of some hydrolytic and oxidative enzymes (Raekallio 1960, 1961) and of transglutaminases (Raekallio & Levenen 1962) has been demonstrated in healing wounds. Among the specific phosphatases adenosine triphosphatase (ATPase) plays an essential role in various metabolic processes. In this investigation therefore, its appearance and distribution in the skin has been studied during the first 24 hours of wound healing.

## MATERIAL AND METHODS

Four month old white rats weighing approx 200 g

Sections were cut at 35  $\mu$  fixed for (Vovkoff et al 1961) washed in one minutes incubated for 15 minutes in (1965) and further processed according to a 51%er phosphate was used instead

## RESULTS

Brown black deposits of lead sulphide indicated ATPase activity in the sections in the unmineralized area.

Most of them  
tate to inten  
cially in the  
laries also showed  
arrectores pilorum muscles were intensely or moderately stained Ac-

This work was facilitated by grants from the Emil Gallenrue Foundation to the authors and from the Vigrid Jurelius Foundation to the department



Fig 1

ATPase activity in an 1 hour-wound. On the right the central zone exhibits a diminishing activity except in the hair follicle. In the peripheral wound zone, the fibroblasts increasingly active in the upper dermis  $\times 150$

tive hair follicles exhibited an intense reaction for ATPase in the upper bulb. The root sheaths were moderately stained. So also were the basal and granular layers of the epidermis. The stratum spinosum showed less conspicuous activity and no reaction at all was observed in the horny layer. In the peripheral cells of the sebaceous glands moderate activity could be seen, whereas it decreased progressively in the cells undergoing sebaceous transformation.

In the immediate vicinity of the injury, the ATPase activity of the dermal fibroblasts and of the capillaries had disappeared in the  $200\mu$  to  $500\mu$  deep central wound zone after only one hour (Fig 1). The activity of the hair follicles, however, was maintained during the experimental period.

Peripherally to the central zone the first signs of increase in the activity of the fibroblasts were noted after one hour (Fig 1). The reaction became more marked in 2- (Fig 2) and 4 hour wounds. After eight hours (Figs 3 and 4) the activated fibroblasts and invading, chiefly polymorphonuclear, leucocytes formed a distinct zone. The leucocytes were more numerous in the vicinity of the panniculus carnosus. In 16- and 24-hour wounds the peripheral zone,  $100$  to  $300\mu$  in depth, consisted of migrant cells, chiefly mononuclears and of intensely active fibroblasts and endothelial cells.

In the panniculus carnosus ATPase activity diminished gradually





Fig. 2

A 2 hour wound ATPase active fibroblasts are seen in the peripheral zone including the lower dermis  $\times 150$

toward the wound edge where the area of necrotizing muscle bundles was occupied by mononuclear cells after 16 to 24 hours.

In the control sections incubated with  $\alpha$  glycerophosphate instead of ATP as substrate strong non specific alkaline phosphatase activity somewhat resembled that of ATPase, but the first signs of increased activity did not appear until after four hours in the peripheral wound zone.

#### DISCUSSION

To the best of our knowledge there has been no previous report concerning the overall distribution of ATPase activity in the intact skin of the rat or any other animal. According to our results, a reaction for ATPase could be demonstrated at the sites of intense metabolic processes, as in the proximal layers of the epidermis, in the upper bulb



Fig. 3

An 8-hour-wound where the invading leucocytes in the lower part contribute to the ATPase activity of the peripheral zone  $\times 150$

and the root sheaths of the active hair follicles, in some fibroblasts, and, of course, in the muscle tissue

In conformity with other enzyme reactions in wounds (Raekallio 1961, 1963), the decrease in ATPase activity in the control zone, nearest to the wound edge, should be considered as an early sign of imminent necrosis. The hair follicles seem to be more resistant than the dermal elements.

An increase in ATPase activity was observed in the peripheral wound zone as early as one hour after the injury. This activity then continued to intensify. It is notable that the fibroblasts were the first to be activated. Montagna (1962) has suggested that the fibroblast is the stem cell from which all other cells of the connective tissue arise, including those with a phagocytic function. According to Senda (1962) phagocytosis is performed by a mechano-chemical system which is connected with ATPase. Thus, the early activation of ATPase in fibroblasts may reflect, *inter alia*, a shift of their function towards phagocytosis. On the other hand, according to Braunstein *et al.* (1962), enzymatic reactions are generally more marked in proliferating cells. In addition, Lennert & Rinneberg (1961) have observed ATPase activity at the sites of vigorous fibre formation. Accordingly, the intensified activity of this



Fig 4

Part of Fig 3 showing the peripheral wound zone with active fibroblasts and leucocytes. In the upper right hand corner of the figure strongly stained capillaries are seen. The ATPase inactive central zone is in the lower right hand corner  $\times 450$

enzyme in the periphery of wounds may be an indication of this proper function of fibroblasts in a later phase of healing. Invasion by leucocytes was clearly demonstrable 8 hours or more after wounding. According to Senda (1962) both the mobility and the phagocytic function of leucocytes are related to ATPase dependent biochemical reactions. This may explain the intense ATPase activity of the invading cells.

In a later phase of healing the non specific alkaline phosphatase and ATPase activities exhibit an almost similar pattern in the wound periphery. At this stage it is difficult to distinguish between these two activities. In the hours immediately subsequent to wounding however the activation of ATPase in the fibroblasts represents the earliest histochemically proved response to injury.

## SUMMARY

Adenosine triphosphatase (ATPase) activity of uninjured and wounded rat skin was studied by using the histochemical method of Padykula & Herman. In the uninjured skin specific ATPase activity was observed in the proximal layers of the epidermis, in the dermal fibroblasts, in the arrectores pilorum muscles and in the prinniculus carnosus. It is apparently present, together with non-specific alkaline phosphatase, in the active hair follicles and in the periphery of the sebaceous glands. In the fibroblasts of the wound periphery a distinct activation of ATPase was noted after only one hour, by which time the enzyme had disappeared from the innermost zone. After eight hours a pronounced peripheral wound zone had been formed containing ATPase-active fibroblast and invading leucocytes.

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## EXPERIMENTAL TOXOPLASMOSIS

### *Parasitemia in guinea-pigs*

By

GUNNAR HULT

Received 2 x 62

In a previous paper (4) it was shown that experimentally infected guinea pigs can transmit toxoplasmosis to their offspring relatively late after inoculation. It was also demonstrated that in two cases *Toxoplasma* could be isolated from the blood 13 and 4 weeks, respectively, after inoculation despite the presence of antibodies in the blood. It seems most likely that the parasitaemia in these cases was transient.

*Toxoplasma gondii* has been demonstrated in the blood of man and different animals by a number of investigators. Studies of parasitaemia in naturally infected pigeons were made in 1949 by *Feldman & Sabin* (2) and in 1952 by *Jacobs* and coworkers (6). In studies of experimental infection in pigeons *Jacobs* and coworkers (7) showed that pigeons contract a heavy parasitaemia which starts 3-8 days after infection and continues until the animal dies, usually 3 weeks after inoculation.

A systematic study of parasitaemia in various mammals was carried out by *Jacobs & Jones* (5) in 1950. The authors showed that in experimentally infected mice and rabbits parasites could be isolated from the blood 4 days after inoculation, and that there was a progressive increase in parasitaemia level until the death of the animal from toxoplasmosis. Mice with asymptomatic toxoplasmic infection exhibited lower blood parasite levels in the early period following inoculation, after which parasites were found in the blood only rarely and in small numbers. A low blood parasite level was also found in one rabbit which survived infection. Experimentally infected rats presented only mild and transient symptoms and showed very low and sporadic parasitaemia.

In studies published in 1961 *Remington, Mellon & Jacobs* (12) showed that parasitemia can occur spontaneously in mice, rabbits and guinea pigs chronically infected with certain strains of *Toxoplasma*.

#### Human parasitemia

In man parasitaemia has been demonstrated in a number of cases (1, 9, 10, 11, 13, 15) of both congenital and acquired toxoplasmosis.

Toxoplasma organisms have been found not only in severe but also in asymptomatic cases of acquired toxoplasmosis (11). In our laboratory Toxoplasma was isolated from the blood in three human cases of acquired toxoplasmosis all of moderate severity (main findings, lymphadenopathy in two cases, myocarditis in one case). In all three cases Toxoplasma was isolated during the first month of the illness, however, antibodies in high titre were found in the same blood sample.

In the present work the parasitaemia of infected guinea-pigs has been studied with respect to the time of its appearance, its duration, and the influence of different infective doses as well as of different modes of infection. Finally, previously infected guinea-pigs have been reinoculated in order to study whether the first infection induced immunity sufficient to protect the animal against new parasitaemia.

## MATERIAL

*Experimental animals.* Seventy-eight guinea pigs were used for the experiments. All animals were young, around 6 months of age (weight 400-500 gm) and of the same breed. They were kept strictly segregated in individual cages and were fed during the experiment only on pellets and Swedish turnips. All lacked antibodies before inoculation according to dye and complement fixation test.

Mice of a well controlled breed free of toxoplasmosis were used for isolation experiments and for providing antigen for the dye test. The mice used for isolation experiments weighed 12-14 gm and for the dye test 16-18 gm.

*Toxoplasma strain.* Sabin's strain RII was used throughout.

## METHODS

*Inoculation of guinea pigs.* Fresh peritoneal exudate from mice with toxoplasmosis was used for inoculating the guinea pigs. The Toxoplasma organisms in the exudate were first counted in a Buerker chamber upon which the exudate was diluted to contain the desired amount of parasites. The inoculum was administered subcutaneously or intranasally (0.2 ml).

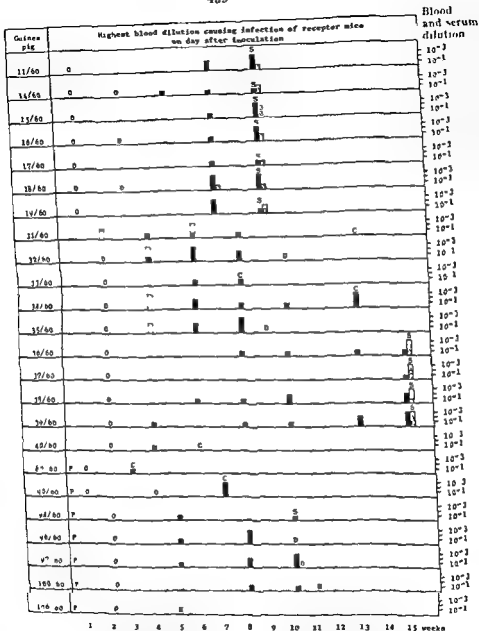
Investigation of the dye test (DT) and the methods employed in this laboratory is paper (4).

The blood was drawn by cardiac puncture. If blood was needed both for serological investigation and for isolation experiments 5 ml were drawn for isolation experiments 2.5 ml were drawn.

*Isolation experiments.* The Sim Aagaard technique (13) was used. The isolations were carried out with heparinized whole blood undiluted and in the dilutions 1/10, 1/100 and 1/10000. 0.5 ml of the material was injected intraperitoneally into mice (5 mice were used for each dilution) immediately after cardiac puncture of the guinea pig.

## EXPERIMENTAL PROCEDURE AND RESULTS

Parasitaemia in experimentally infected guinea-pigs was studied in five separate experiments. Three main problems were investigated namely the appearance and early development of parasitaemia in DT and CFT-negative animals (group A, B), the duration of parasitaemia in such animals (group C, D), and finally the protective effect against parasitaemia obtained by immunization with living parasites (group E).



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0 = isolation experiment negative
1 = isolation experiment positive
2 = isolation experiment positive only
  of the bare indicated by the top
3 = DT negative
4 = DT titer

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KI = CFT negative  
EJ = CFT titer  
B = spontaneously dead  
C = dead by cardiac puncture  
S = sacrificed  
AC = serum anticomplementary  
T = peritoneal

Fig. 1

Group A  $\square$  guinea pigs inoculated subcutaneously with 5 000-10 000 toxoplasmas  
mice and serum dilutions on log scale

## Group A

Parasitaemia during the first two weeks after inoculation was followed. For this purpose 24 guinea pigs, 7 of which were pregnant, were inoculated with 5,000–10,000 toxoplasma organisms. Isolation experiments on heart blood and dilutions were made every two days (on a few occasions every 3 days) on the non-pregnant animals, beginning with the first day in 9 animals and with the second day in 8 animals. In the pregnant guinea-pigs isolation experiments were carried out less frequently (every 3 days) because of the sensitivity of these animals to cardiac puncture. During the experiment 12 animals died. The remaining 12 animals were sacrificed, 7 on the ninth day after inoculation, one on the tenth day, and 4 on the fifteenth day. Immediately before the animals were killed, blood for isolations, DT, and CFT was taken.

Fig. 1 shows the appearance of *Toxoplasma* in the blood and the blood parasite level in the individual animals. The DT and CFT in blood taken immediately before the animals were sacrificed is also demonstrated. One of 15 animals investigated had parasitaemia on the second day after inoculation, 5 of 7 on the third day, and 10 of 11 on the fourth day. On the fifth day *Toxoplasma* was isolated from all of the blood samples in dilutions 1/1–1/1000. In 5 isolation experiments *Toxoplasma* was detected in one high blood dilution whereas one or two lower dilutions were negative. This phenomenon will be discussed later. The blood

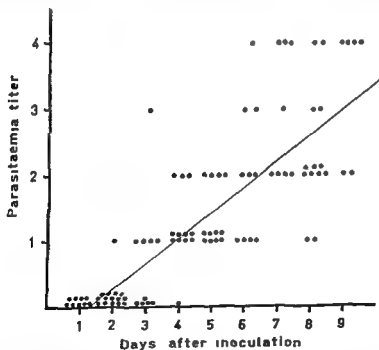


Fig. 2

Guinea pigs group A. Blood parasite level on days after inoculation. Ordinate: Dilutions of blood from which toxoplasma were isolated. Blood dilutions on log scale. Significance of symbols are indicated in the text.



parasite level in these cases is considered to be represented by the highest positive dilution with all the lower dilutions positive.

In Fig. 2 the individual isolations are recorded. The diagram covers only 9 days of the experiment, because after the ninth day only 9 of 22 animals were alive, the material was therefore no longer sufficiently representative. It can be seen from the diagram that an increase in blood parasite level set in on the third day after inoculation and proceeded during the time recorded. This increase is highly significant, the variation quotient,  $V^2 = 16.2$ ,  $p < 0.001$ .

From Fig. 1, it can be seen that parasitaemia did occur despite the presence of antibodies in high titre in the blood. This was better demonstrated in groups C and D.

In group A no marked difference in parasitaemia level was seen between animals with clinical and subclinical toxoplasmosis in contrast to Jacob's findings in mice and rabbits. Of the three non-pregnant animals that died spontaneously two (32.60, 38.60) had parasitaemia in high titre while in the third (10.60) parasitaemia could be demonstrated only in low titre. On the other hand high titres were demonstrated in animal 34.60 which showed no signs of disease during the experiment. However the numbers are insufficient to permit further conclusions in this respect.

Parasitaemia in pregnant guinea pigs followed the same pattern as in non-pregnant animals.

### Group B

In order to investigate whether a small infective dose might yield a lower degree of parasitaemia, a group of 9 guinea pigs were injected with a much smaller dose than that used in group A. 100-200 parasites were injected subcutaneously in each of 9 guinea pigs. Isolation experiments on heart blood were made every second day beginning with the first day after inoculation. Eight animals died within the interval from day 7 to day 13 of the experiment. One animal was sacrificed on the fourteenth day after inoculation. Serological investigation after inoculation was not carried out in this group.

From Fig. 3 can be seen that in all cases *Toxoplasma* was isolated after inoculation, at which time the seventh day *Toxoplasma* is. The blood parasite level reached the same values in this group as in experimental group A, in which the animals had been given a heavier infective dose.

In Fig. 4 the individual titre values on different days after inoculation are recorded. It can easily be seen that a marked increase in blood parasite level began on the fifth day after inoculation and continued during the time covered by the diagram i.e., 11 days. Highly significant values were also found in this material.  $V^2 = 17.5$   $P < 0.001$ . There is

## Group A

Parasitaemia during the first two weeks after inoculation was followed. For this purpose 24 guinea pigs, 7 of which were pregnant, were inoculated with 5,000–10,000 toxoplasma organisms. Isolation experiments on heart blood and dilutions were made every two days (on a few occasions every 3 days) on the non-pregnant animals, beginning with the first day in 9 animals and with the second day in 8 animals. In the pregnant guinea-pigs isolation experiments were carried out less frequently (every 3 days) because of the sensitivity of these animals to cardiac puncture. During the experiment 12 animals died. The remaining 12 animals were sacrificed, 7 on the ninth day after inoculation, one on the tenth day, and 4 on the fifteenth day. Immediately before the animals were killed, blood for isolations, DT, and CFT was taken.

Fig. 1 shows the appearance of *Toxoplasma* in the blood and the blood parasite level in the individual animals. The DT and CFT in blood taken immediately before the animals were sacrificed is also demonstrated. One of 15 animals investigated had parasitaemia on the second day after inoculation, 5 of 7 on the third day, and 10 of 11 on the fourth day. On the fifth day *Toxoplasma* was isolated from all of the blood samples in dilutions 1/1–1/1000. In 5 isolation experiments *Toxoplasma* was detected in one high blood dilution whereas one or two lower dilutions were negative. This phenomenon will be discussed later. The blood

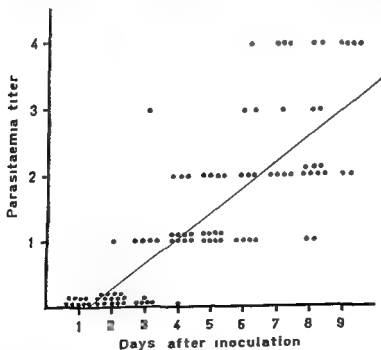


Fig. 2

Guinea pigs group A. Blood parasite level on days after inoculation. Ordinate: Dilutions of blood from which toxoplasma were isolated. Blood dilutions on log scale. Significance of symbols are indicated in the text.

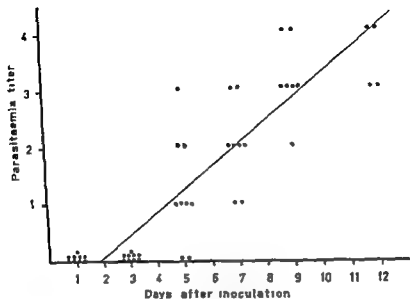


Fig. 4

Guinea pigs group B. Blood parasite level on days after inoculation. Ordinate: Dilutions of blood from which toxoplasma were isolated. Blood dilutions on log scale. Significance of symbols are indicated in the text.

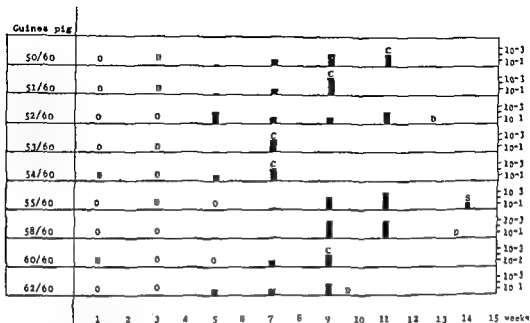
Toxoplasma was isolated from the blood of only one of 12 animals investigated. As from the fourth week transient parasitaemia was occasionally demonstrated in two animals. In one case, in which isolations from the blood had been negative for seven weeks, Toxoplasma was isolated from the blood 15 weeks after infection (Fig. 5).

Compared with groups A and B the blood-parasite titres in group C showed lower values throughout. In most cases the blood-parasite level was highest in samples taken one week after inoculation. The transient parasitaemia occasionally appearing after the third week did not reach higher titres than 1:1 except in 2 cases. 439, in which the titre was 1:100 7 weeks after inoculation, and 2459 in which the titre was 1:100 3 weeks after inoculation.

From Fig. 5 it can be seen that in several cases Toxoplasma could be isolated from blood containing antibodies in high titre. No marked difference in blood-parasite level between clinical and subclinical cases of toxoplasmosis can be seen in this material either.

#### Group D

The experiments were performed one year earlier than those of the preceding group. The main purpose was to study the duration of parasitaemia after intracutaneous and intranasal inoculation.



- = isolation experiment negative  
 ■ = isolation experiment positive  
 D = spontaneously dead  
 C = dead by cardiac puncture  
 S = sacrificed

Fig 3

Group B 9 guinea pigs inoculated subcutaneously with 100-200 toxoplasmas  
Blood dilutions on log scale

no important difference between the diagrams in Figs 2 and 4 except the later appearance of parasitaemia in Fig 4 where the animals had obtained a smaller infective dose

A comparison between the blood parasite level in cases with clinical and subclinical toxoplasmosis does not show any obvious differences. Guinea-pig 58/60 which died spontaneously had high parasitaemia titres in the last two blood samples. However, titres of the same value were found in animal 55/60 which did not show any signs of disease. Moreover, in the animals 52/60 and 62/60 which also died spontaneously Toxoplasma was isolated from the blood only in low and moderate titres.

### Group C

The aim of this experiment was to study the duration of parasitaemia. Seventeen guinea-pigs were injected subcutaneously with 5,000-10,000 parasites. Isolation experiments were carried out on heart blood and dilutions thereof once every week after inoculation, starting 7 days after inoculation in 13 animals and 14 days in 4 animals. DT, CFT, and isolations were carried out on the same blood sample. During the first 7 weeks 15 animals died from heart puncture or disease. The two surviving animals were sacrificed 7 and 15 weeks after inoculation.

One week after inoculation all investigated animals had parasitaemia. After two weeks 9 of 17 animals had parasitaemia. After three weeks

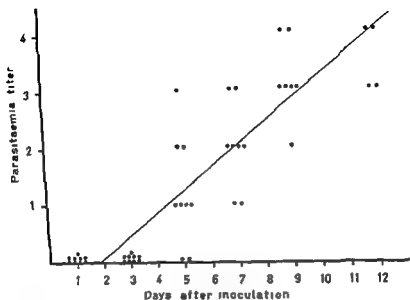


Fig 4

Guinea pigs group B Blood parasite level on days after inoculation Ordinate Dilutions of blood from which toxoplasma were isolated Blood dilutions on log scale Significance of symbols are indicated in the text

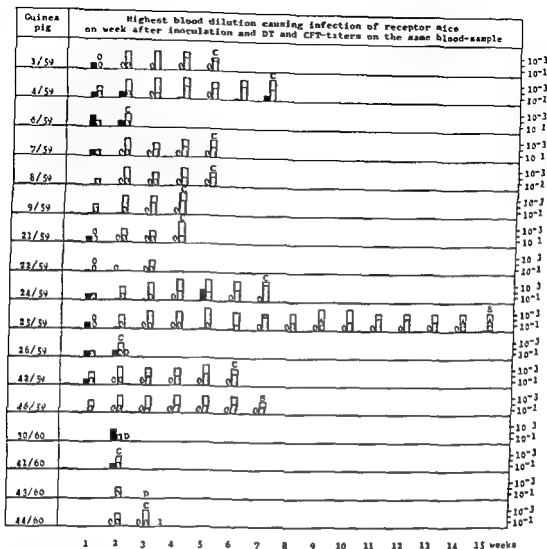
Toxoplasma was isolated from the blood of only one of 12 animals investigated. As from the fourth week transient parasitaemia was occasionally demonstrated in two animals. In one case, in which isolations from the blood had been negative for seven weeks, *Toxoplasma* was isolated from the blood 15 weeks after infection (Fig 5).

Compared with groups A and ■ the blood-parasite titres in group C showed lower values throughout. In most cases the blood parasite level was highest in samples taken one week after inoculation. The transient parasitaemia occasionally appearing after the third week did not reach higher titres than 1/1 except in 2 cases: 4/59, in which the titre was 1/100 7 weeks after inoculation, and 24/59 in which the titre was 1/100 5 weeks after inoculation.

From Fig 5 it can be seen that in several cases *Toxoplasma* could be isolated from blood containing antibodies in high titre. No marked difference in blood parasite level between clinical and subclinical cases of toxoplasmosis can be seen in this material either.

#### Group D

The experiments were performed one year earlier than those of the preceding group. The main purpose was to study the duration of parasitaemia after intracutaneous and intranasal inoculation.



0 = isolation experiment negative  
 ■ = isolation experiment positive  
 □ = DT titer  
 ▤ = CFT negative  
 ▥ = CFT titer  
 D = spontaneously dead  
 C = dead by cardiac puncture  
 S = sacrificed

Fig 5

Group C 17 guinea pigs inoculated subcutaneously with 5 000 10 000 toxoplasmas  
Blood and serum dilutions on log scale

Three animals were injected intracutaneously with 0.1 ml of a suspension containing 100,000–800,000 parasites per ml (infective dose 40,000–80,000 toxoplasmas)

In seven animals 0.1 ml of a suspension containing 200,000–400,000 toxoplasmas per ml was administered into each nostril. Thus the infective dose given to the intracutaneously infected animals and to those infected intranasally was the same.

Isolation experiments were made only on undiluted blood

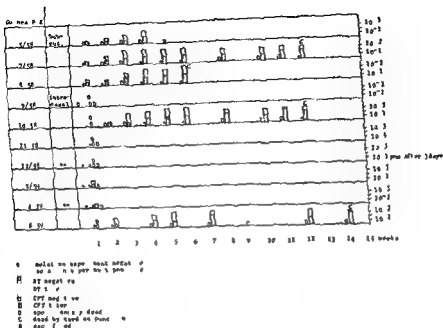


Fig 6

Guinea Pig 3 guinea pigs inoculated subcutaneously 7 guinea pigs inoculated intranasally all are males with 400 000-500 000 toxoplasmas Serum dilutions on log scale

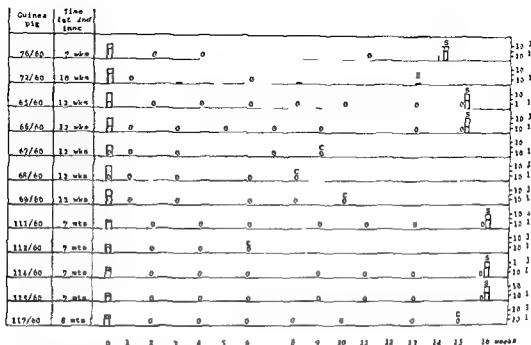
In 5 of 7 intranasally infected animals *Toxoplasma* was isolated from the blood 3 days after inoculation, that is at the same time as would be expected after subcutaneous inoculation. Concerning the duration of parasitaemia the results agree in the main with those obtained in the preceding group. It is obvious even in this small material (Fig 6) that parasitaemia can occur in the presence of antibodies in high titre in the blood (8.08). In one case *Toxoplasma* could be isolated from the blood 12 weeks after inoculation (10.08).

### Group E

The possible appearance of parasitaemia in animals immunized with living parasites was studied in this experiment.

In all 18 animals were injected subcutaneously with 5 000-10 000 toxoplasmas. Six animals died during the second week after inoculation. The surviving 12 animals were given the same infective dose by the same route at different times between 7 weeks and 8 months later. Blood samples for isolation experiments were taken every second or third day during the first 2 weeks after the second inoculation. During the experiment 6 guinea pigs died upon cardiac puncture. The remaining 6 animals were sacrificed 14-16 weeks after inoculation.

From Fig 7 it can be seen that during the experiment *Toxoplasma*



- O = isolation experiment negative  
 ■ = isolation experiment positive  
 □ = isolation experiment positive only in dilution indicated by the top of the bar  
 ⊞ = DT titer  
 ⊙ = CFT negative  
 ⊕ = CFT titer  
 ⊖ = dead by cardiac puncture  
 ⊗ = sacrificed

Fig 7

Group 12 previously immunized guinea pigs inoculated subcutaneously with 5 000–10 000 toxoplasmas. Blood and serum dilutions on log scale.

was isolated from the blood only in 2 cases. These two animals had received their first inoculation 7 and 10 weeks earlier, respectively, and both had antibodies in high titre in the blood. It is impossible to know whether the parasitaemia in these cases is referable to the first or to the second inoculation.

In the remaining guinea-pigs *Toxoplasma* was not isolated from the blood during the first weeks after inoculation, despite the fact that in several cases, especially those which had been immunized 7 or 8 months earlier, antibody titres had decreased to low values. In two cases (67/60, 68/60) the CFT was negative before the second inoculation.

### Clinical Symptomatology and Death Rate

Fifty-seven of the 78 inoculated animals died during the course of the experiment. 33 deaths occurred during or immediately after cardiac puncture; the remaining 24 animals died spontaneously. There is reason to believe that toxoplasmosis was the cause of death in most of the cases. No animal presented signs of any other disease. Furthermore, *Toxoplasma* was isolated from the blood in all of the 18 cases.



investigated for parasitaemia. Finally the majority, 22 animals, died 10-16 days after inoculation which corresponds to the survival time established in guinea pigs (4) becoming seriously affected.

TABLE 1  
*Death Rate and Signs of Diseases in Guinea Pigs Belonging to the Different Experimental Groups*

Group	Number of animals	Number of deaths			Mortality		Number of animals with signs of diseases
		Spontaneous	Card punct.	Sacrificed	Spontaneous	Card punct.	
A	24	5	7	12	20.8	29.1	2
B	9	3	5	1	33.3	55.6	1
C	17	4	11	2	23.5	64.7	3
D	10	6	4		60.0	40.0	8
E	18	8	6	4	33.3	33.3	
Total	78	24	33	21	30.8	42.3	14

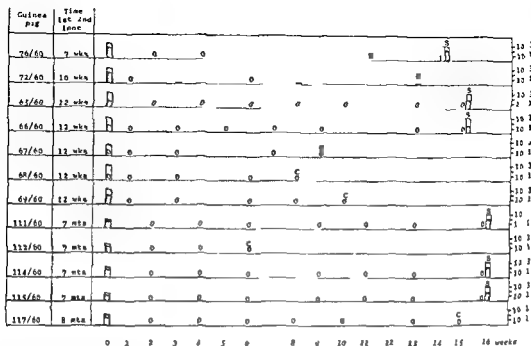
Table 1 shows the distribution of deaths in the different groups and the occurrence of clinical signs in the animals. Fourteen animals developed visible signs of disease. All of the 7 intranasally infected guinea-pigs had running noses, cough, and lymphadenopathy of the neck glands. Five of these died during the second week after inoculation, the remaining two recovered. Eight of the subcutaneously infected -

... also observed five of these animals died and 3 recovered. Fourteen animals were found dead in their cages without having exhibited signs of disease.

It can be seen from table 1 that the mortality from toxoplasmosis was considerably greater in group D than in the other groups. Most of the deaths in this group occurred among intranasally infected animals.

The total mortality in toxoplasmosis in all of the groups is 20.8 per cent as compared with 32 per cent in an earlier investigation (4). However, the figures must be considered as approximate, partly because death occasionally may have been caused by more than one disease and partly since it cannot be precluded that some of the 33 animals which died in connection with heart puncture may have been seriously affected by toxoplasmosis, and hence would have succumbed to its disease.

The high rate of deaths ascribable to cardiac puncture (42.3 per cent of the whole material) is of course regrettable. However, it has been impossible to avoid deaths of animals so often deprived of a considerable part of their total blood volume. The repeated injuries to the heart muscle may also have played a rôle.



- = isolation experiment negative  
 ● = isolation experiment positive  
 ◐ = isolation experiment positive only in dilution indicated by the top of the bar  
 □ = CFT negative  
 ■ = CFT positive  
 C = dead by cardiac puncture  
 S = sacrificed

Fig 7

Group I: 12 previously immunized guinea pigs inoculated subcutaneously with 5 000–10 000 toxoplasmas. Blood and serum dilutions on log scale

was isolated from the blood only in 2 cases. These two animals had received their first inoculation 7 and 10 weeks earlier, respectively, and both had antibodies in high titre in the blood. It is impossible to know whether the parasitaemia in these cases is referable to the first or to the second inoculation.

In the remaining guinea-pigs *Toxoplasma* was not isolated from the blood during the first weeks after inoculation, despite the fact that in several cases, especially those which had been immunized 7 or 8 months earlier, antibody titres had decreased to low values. In two cases (67/60, 68/60) the CFT was negative before the second inoculation.

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B and parasitaemia developing in animals in group C which were examined less frequently but for a longer period. This difference has probably more than one explanation. It is to be expected that the parasitaemia gradually decreases with time; this would be reflected in the less frequent detection of the parasites in high titre in a more protracted study. It is also probable that the considerable loss of blood caused by the frequent cardiac punctures in animals belonging to groups A and B contributes to the occurrence of more pronounced parasitaemia in these animals. The fact that all of the 27 investigated animals in groups A and B presented parasitaemia in the second week after infection as opposed to 9 only of 17 animals in group C presenting a parasitaemia 2 weeks after inoculation also points in this direction.

A phenomenon appearing in several isolation experiments in different experimental groups is that *Toxoplasma* sometimes was isolated in a higher dilution of blood whereas isolation experiments from lower dilutions of the same blood sample were negative. Probably more than one factor is responsible. A probable explanation is that *Toxoplasma* may be present intracellularly in the blood. Intracellular parasitaemia with the appearance of *Toxoplasma* in *e.g.* elements belonging to the white blood cell system can be expected to give erratic results in the isolations because in such cases more than one parasite is located in a single cell.

However, the fact which above all points to the occurrence of intracellular parasitaemia is the simultaneous appearance of both *Toxoplasma* and antibodies in high titre in the same blood sample. The transient parasitaemia demonstrated in some animals after 3 weeks is also most easily explained as a result of the occasional appearance in the blood of one or a few *Toxoplasma* infected cells.

Experiment I shows that a thorough *Toxoplasma* infection gives immunity against re-induced parasitaemia for at least 8 months. This experiment also demonstrates that a real parallelism between DT and CFT titres and immunity does not exist. More than one of the immune animals had comparatively low titres. This question will be elucidated more closely in a coming investigation.

Immunity in toxoplasmosis is complicated and for the most part still obscure. As in infections with other intracellular parasites.

## SUMMARY

Two  
groups  
inoculated

Toxo  
were

Sixty animals seronegative at the start of the experiments were

### *Serological investigation*

Combining the results from the different groups the conclusions to be drawn are the same as the ones drawn in an earlier investigation on guinea pigs (4) DT proved to be positive at an earlier stage than CFT. One week after inoculation DT was positive in 18 of 29 investigated samples, while CFT was negative in all of the samples. Three weeks after inoculation DT was positive in the dilution 1/250 or above in all of the 16 animals investigated, while CFT remained negative in 11 of 16 examined animals. It was not until the sixth week that all of the investigated animals were positive using CFT, however, and CFT values above 1/120 could not be revealed until 7 weeks after inoculation.

### DISCUSSION

The experiments show that parasitaemia regularly appeared in guinea-pigs experimentally (subcutaneously, intracutaneously or intranasally) infected with *Toxoplasma* if the animals were seronegative before inoculation. Parasitaemia could be demonstrated as early as 3-5 (in one case, 2) days after inoculation, and it persisted for 1-3 (most commonly 2) weeks. After this time transient parasitaemia could be demonstrated in some cases as late as 15 weeks after inoculation.

The results indicate that the parasites do not enter the blood before initial multiplication has taken place. This suggestion is supported by the fact that the period between inoculation and the development of parasitaemia was longer when the infective dose was smaller. Furthermore, the early enlargement of the regional lymph glands in the intranasally infected guinea-pigs points to a primary multiplication of the parasites in the glands. Relevant questions will be elucidated in a coming article.

An attempt was made to estimate the degree of parasitaemia by determining the highest dilution of 0.5 ml of blood (within the limits 1/1-1/1000) causing toxoplasmosis in mice. With the aid of this method a statistically significant continuous increase in the degree of parasitaemia was demonstrated during the first 3-9 days in animals injected with a medium sized infective dose. A similar continuous increase was demonstrated during the first 5-11 days in animals injected with a small infective dose. The gradual increase in parasitaemia supports the concept of a primary local multiplication of the parasites. It is remarkable in this disease that the parasitaemia can persist for as long as 2-3 weeks. No difference was demonstrable in the degrees of parasitaemia to develop in animals with clinical and with asymptomatic toxoplasmosis. It is difficult to give an explanation of this fact. However, it is obvious that the number of circulating parasites is not an adequate characteristic of the severity of the disease.

There is a marked difference in degrees of parasitaemia developing in the more intensively investigated animals belonging to groups A and



examined with respect to the occurrence of parasitaemia after inoculation. In all animals parasitaemia could be demonstrated.

Parasitaemia was demonstrated earliest 3-5 (in one case, 2) days after inoculation. When the infective dose was of medium size the parasitaemia appeared on the third to fourth day, when the dose was small, on the fifth day.

Parasitaemia usually continued for 1-3 (most commonly 2) weeks, after which transient parasitaemia could be demonstrated in some cases as late as 15 weeks after inoculation.

It could be demonstrated that the degree of parasitaemia increases continuously for 6 days after it was first detected.

Toxoplasma organisms and Toxoplasma antibodies in high titre have been demonstrated in the same blood sample on several occasions. This observation supports the idea that Toxoplasma can exist intracellularly in the blood.

Finally, the investigation shows that a thorough Toxoplasma infection in guinea pigs can produce immunity against the reinduction of parasitaemia for at least 8 months.

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TABLE 1  
Testing of  $\epsilon$  Antigen against 5 Pools of *Corynebacterium* *Hyperimmune* Sera

Antigen	Dilutions	Hyperimmune serum pools*										Normal serum	Antigen control	CF reaction with pool No.	Result of neutral tests	H <sub>2</sub> O of $\epsilon$ P antigen neg. log
		1*	1/2	1/3	1/4	1/5	1/6	1/7	1/8	1/9	1/10	1/11	1/12			
3088	1	+	+	+	+	+	+	+	+	+	+	+	+	0 (1 n s t)	A 16	3.5
2	1	0	0	0	0	0	0	0	0	0	0	0	0	I	A 4	ca 7.1
3171	1	+	+	+	+	+	+	+	+	+	+	+	+	IV (1 n s)	A 16	4.0
2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3420	1	+	+	+	+	+	+	+	+	+	+	+	+	II (1 n s)	A 8	7.1
2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3614	1	+	+	+	+	+	+	+	+	+	+	+	+	I III	1.5	7.8
2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4373	1	+	+	+	+	+	+	+	+	+	+	+	+	I	A 1	ca 6.8
2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4617	1	+	+	+	+	+	+	+	+	+	+	+	+	II (1 n s)	A 10	6.6
2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4317	1	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0
2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5432	1	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0
2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Serum control	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

\* Dilutions of individual sera in all pools 1:5 and 1:20

† Reciprocal of serum or antigen dilutions (1 → undiluted) 2 = 1:2, 6 = 1:6, 16 = 1:16, etc.

III = non-specific

Other legends see text

Synchronous complement titration in Veronal buffer

Units of complement	2	15	1	0.75	0.5
Reaction grade	0	0	(+)	+	1

*Preparation of CF antigen* The method of antigen preparation has been presented in detail in preceding papers (1,2) and shall only briefly be reviewed here. Two litters of newborn mice usually less than 24 hours of age were inoculated intraperitoneally with 0.02 ml of a 10 per cent stool suspension to which penicillin and streptomycin had been added. The animals were observed daily for 14 days for typical signs of illness and diseased mice were killed by ether.

In order to increase the virus titer, one additional mouse passage of the virus was routinely carried out in two litters of newborn mice using a 10 per cent torso suspension as passage material. All diseased animals were killed by ether and stored at  $-20^{\circ}\text{C}$ . For antigen preparation a 20 per cent suspension of the torsos was prepared and purified with Freon 112, followed by addition of guinea pig serum and inactivation. The antigens were stored at  $-20^{\circ}\text{C}$  and centrifuged before use.

*Virus titrations* were performed in newborn mice as described earlier (1).

*Complement fixation tests* All details have been given in earlier papers (1,2).

Since the Coxsackie viruses A 20 through 24 were not available to us until late in this study, testing of the CF antigens against hyperimmune sera of these virus types were not included.

## EXPERIMENTAL AND RESULTS

*Typing of "unknown" antigens* was performed in two steps. First the antigens were examined in CF tests against pools of hyperimmune mouse sera. Whenever an antigen was found to react with one of the serum pools, the next step was to test the antigen against the individual sera contained in the pool.

It was studied whether typing of antigens could always be done employing antigens undiluted or diluted 1:2. Undiluted antigens were used to ensure maximal amounts of antigen in the test, in case the potency of preparation was low. A 1:2 dilution was included with the possibility in mind that a concentrated antigen may give rise to a prozone phenomenon, or possibly to non-specific reactions.

In order to simplify the tests, and to save antigens and sera, titrations of complement in the presence of the dilutions of antigens and sera employed in the tests proper were as a rule omitted. This was found justifiable since experience gained in numerous previous experiments had shown that anticomplementary activity of the kind of antigen and antisera preparations employed was so slight that it did not interfere with the reading of the results (1). A control titration of complement in veronal buffer was always included in the experiments.

As regards reading of the CF tests, fixation grades of 3 or 4 with one or more of the antigen- and serum dilutions examined were recorded as positive reactions. Whether fixation grades of 2 or less were recorded as specific was determined by comparison with the results obtained with the other serum types and with the controls included in the tests. If equivocal reactions were obtained with an antigen, the test was repeated, and if the results were still equivocal it was attempted to prepare a more potent CF antigen using third mouse passage material for the preparation.

*CF typing experiments employing low dilutions of antisera* When the first experiments in this study were performed, the exact CF titers of all hyperimmune sera had not yet been determined. Serum pools—each



containing equal amounts of 5 different types of hyperimmune sera—were therefore used undiluted and diluted 1/10 this representing dilutions of 1/5 and 1/30 respectively of the individual sera in the pools. In some of the pools normal mouse serum was added to obtain these dilutions. The same two dilutions i.e. 1/5 and 1/30 of the individual sera were used during the second step of the experiments.

In all the tables in this paper the results of typing of the virus isolates by neutralization tests in newborn mice are listed for comparison with the CF results. The infectious titers of the antigens have also been recorded. These data will be discussed later in the paper.

In Table 1 results obtained with 11 antigens in the first step of a CF typing experiment are recorded. It will be seen that all the antigens except No. 3088 gave a positive reaction with the pool containing the homologous hyperimmune serum and with this pool only. A slight fixation was encountered between all the undiluted serum pools and all the undiluted antigens, this being especially pronounced for pool I which also gave weak reactions with antigens diluted 1/2. However, experience from previous experiments (1) allowed the interpretation of these reactions as being non specific.

A slight crossing was found between antigen No. 4353 and serum pool III containing hyperimmune sera A 11 through 15. Since this antigen had previously been typed in neutralization tests as a Coxsackie A 5 strain this reaction could be reasonably ascribed to the cross reactions known to exist between types A 5 and A 12 (1).

Table 2 presents the results obtained when the 7 reacting antigens from Table 1 were tested against the individual sera in the reacting pools. As will be seen each CF antigen was found to react with the homologous serum only.

TABLE 3

Testing of Coxsackie A CF Antigens Prepared from Second and Third Mouse Passage Material

Antigen No.	Lot	Hyperimmune serum pools					Reciprocal dilution	CF reaction with pool No.	Result of neutral tests	ID <sub>50</sub> of CF antigen neg. log
		I	II	III	IV	V				
		1/5	1/10	1/15	1/20	1/30				
5913 2 pass	1	2/1	0/0	0/0	0/0	0/0	0	1/2	A 1	ca 6.6
	2	1/4	0/0	0/0	0/0	0/0	0			
5913 3 3 pass	1	3/3	0/0	0/0	0/0	0/0	0	1	A 1	7.2
	2	3/3	0/0	0/0	0/0	0/0	0			
Serum control		0/0	0/0	0/0	0/0	0/0	0			

Reciprocal of serum or antigen dilutions  
(Antigen—see Table 1)

Simultaneous complement titration in Veronal buffer					
Units of complement	1/5	1	0.75	0.5	
Fixation grade	0	0	0	+	+

TABLE 2  
Testing of CP Antigens from Table 1 against Individual Sera Contained in the Reacting Serum Pools

Antigen	Convalescent & hyperimmune sera																				Antig con trol	Result of neutral tests	ID <sub>50</sub> of CI antigen neg log																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						
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No	Dilut	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4

\* not done

• Reciprocal of serum or antigen dilutions

Simultaneous complement titration in Veronal buffer  
Units of complement = 1 5 1 0 75 0 5  
Fixation grade 0 0 + + + 1

containing equal amounts of 5 different types of hyperimmune sera—were therefore used undiluted and diluted 1/6 this representing dilutions of 1/5 and 1/30 respectively, of the individual sera in the pools. In some of the pools normal mouse serum was added to obtain these dilutions. The same two dilutions, i.e. 1/5 and 1/30, of the individual sera were used during the second step of the experiments.

In all the tables in this paper the results of typing of the virus isolates by neutralization tests in newborn mice are listed for comparison with the CF results. The infectious titers of the antigens have also been recorded. These data will be discussed later in the paper.

In Table 1 results obtained with 8 antigens in the first step of a CF typing experiment are recorded. It will be seen that all the antigens except No. 3088 gave a positive reaction with the pool containing the homologous hyperimmune serum and with this pool only. A slight fixation was encountered between all the undiluted serum pools and all the undiluted antigens, this being especially pronounced for pool I which also gave weak reactions with antigens diluted 1/2. However, experience from previous experiments (1) allowed the interpretation of these reactions as being non specific.

A slight crossing was found between antigen No. 4353 and serum pool III containing hyperimmune sera A 11 through 15. Since this antigen had previously been typed in neutralization tests as a Coxsackie A 5 strain this reaction could be reasonably ascribed to the cross reactions known to exist between types A 5 and A 12 (1).

Table 2 presents the results obtained when the 7 reacting antigens from Table 1 were tested against the individual sera in the reacting pools. As will be seen each CF antigen was found to react with the homologous serum only.

TABLE 3  
Testing of Coxsackie A CF Antigens Prepared from Second and Third Mouse Passage Material

Antigen	Hyperimmune serum pools					Atlig control	CF reaction with 1:10	Result of neutral tests	ID <sub>50</sub> of CF antigen neg log
	I				II				
	1:5	1:6	1:10	1:15	1:15				
No.	Dilut.	1:5	1:6	1:10	1:15				
5913 2 pass	1	2/1	0/0	0/0	0/0	0	1/2	A 1	ca 6.6
	2	1/4	0/0	0/0	0/0	0			
5915 3/3 pass	1	3/3	0/0	0/0	0/0	0	1	A 1	7.2
	2	3/3	0/0	0/0	0/0	0			
Serum = ntr 1		0/0	0/0	0/0	0/0	0			

Reciprocal of serum or antigen dilutions  
Legends: see Table 1

Simultaneous complement titration in Verona buffer					
Units of complement	2	1.5	1	0.75	0.5
Fixation grade	0	0	0	+	+

TABLE 4

Testing of CI Antigens from Table 3 against Individual Coxsackie A Antisera Containing in the Reacting Serum Pool

Antigen		Hyperimmune serum pool I										Antig control	Result of CI tests
		A 1		A 2		A 3		A 4		A 5			
No	Dilut	5*	30	5	30	5	30	5	30	5	30		
5913, 2 pass	1*	3	3	0	0	0	0	0	0	0	0	0	A 1
	2	2	1	0	0	0	0	0	0	0	0	0	
5913 7 3 pass	1	4	4	0	0	0	0	0	0	0	0	0	A 1
	2	4	4	0	0	0	0	0	0	0	0	0	
Serum control	-	0	0	0	0	0	0	0	0	0	0	0	

\* Reciprocal of serum or antigen dilutions

Simultaneous complement titration in Veronal buffer

Units of complement	2	1.5	1	0.75	0.5
Fixation grade	0	0	+	1	1

TABLE 5

Testing of CI Antigens against 4 Pools of Coxsackie A Hyperimmune Sera

Exp No	Antigen No.   Dilut		Hyperimmune serum pools*				Antig control	CI reaction with pool No	Result of neutral tests	H <sub>50</sub> of CI antigen neg l <sub>50</sub>
			I	II	III	IV				
348	6706	1§	4	0	1	0	+	I III	A 5	7.9
		2	3	0	1	0	0			
	6725	1	3	0	1	0	+	I	A 2	7.0
		2	2	0	0	0	0			
357 A	6845	1	1	0	+	0	0	I	A 1	ca 6.0
		2	+	0	0	0	0			
	Control	1	1	0	+	0	0	IV	A 16	5.5
		2	+	0	0	0	0			
357 B	6934	1	+	0	0	3	+	IV ?	A 16	5.5
		2	+	0	0	2	0			
	7119	1	0	0	0	1	0	IV ?	A 16	5.5
		2	0	0	0	+	0			

\* 1:20

Simultaneous complement titrations in Veronal buffer

Exp No	Units of complement				
	2	1.5	1	0.75	0.5
348	0	0	0	0	(+)
357 A	+	+	+	+	+
357 B	0	0	0	0	+
387	0	0	0	0	0

TABLE 6  
Testing of 18 Antigens from Table 5 against Individual Sera Contained in the Reacting Serum Pools

Testing of 18 Antigens from Table 3 against various strains

Exp No	Antigen	Coarsely & hyperimmune sera																		Result of neutral test	ID of CT antigen neg log
		Food I			Food III						Food IV						Antig control	Result of FLI test			
		A 1 10 <sup>+</sup>	A 2 10 <sup>+</sup>	A 4 12 <sup>+</sup>	A 10 12 <sup>+</sup>	A 11 16	A 12 16	A 13 16	A 14 12 <sup>+</sup>	A 15 32	A 16 32	A 17 15	A 18 16	A 19 16							
358	5706	1 <sup>+</sup>	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	A 5	A 5	7.9
	2	0	0	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	A 2	A 2	7.0
	6725	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	A 1	A 1	6.0
	2	0	0	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	A 1	A 1	6.0
6845	1	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	A 10	A 10	5.5
	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	A 10	A 10	5.5
	6954	1																			
	2																				
388	Serum control		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	A 16	A 16	5.5
	7119	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			

- = not done

\* Reciprocal of serum or antigen dilutions

Simultaneous complement titrations in Veronal buffer

Units of complement				
Exp No	2	1.5	1	0.75
358	0	0	0	0
388	0	0	0	0

TABLE 7

*Summary of Complement Fixation Tests with Antigens Prepared from Second Mouse Passage of Coxsackie A Virus Strains Recovered from Stool Specimens*

Type* of antigen	Number of antigens exam	Number of antigens typed	Antigens not reacting	$\log$ of reacting antigens neg log	$\log$ of non reacting antigens neg log
Coxs A 1	6	5	1	6.0 6.8	4.9
- A 2	1	1	0	7.0	
- A 4	2	2	0	7.1-8.0	
- A 5	5	5	0	7.5 7.9	
- A 8	3	3	0	7.0 7.6	
- A 10	1	1	0	6.6	
- A 16	5	4	1	4.9-5.5	3.5
Total	23	23	2		

\* Type as determined by neutralization tests in newborn mice

Tables 3 and 4 show the difference in reactions obtained when a weak antigen No 5913 prepared from second mouse passage material was compared to the more potent antigen, No 5913-3, prepared from third mouse passage of the virus strain. It can be seen that although the reactions encountered between serum pool I and antigen No 5913 were not much stronger than the non-specific reactions occasionally obtained with this serum pool, they were in this case nevertheless specific. Table 4 shows that stronger reactions were obtained in the second step of the typing. The control titrations of complement indicate that variations in the amount of complement present in the tests account for these differences in fixation grades.

*CF typing experiments employing higher dilutions of antisera.* When later in this study the CF antibody titers of all the types of hyperimmune sera had been determined, it was attempted to employ higher serum dilutions in order to save sera and to avoid non-specific reactions occasionally seen with more concentrated sera. Depending on the 'average' level of the CF titers of the individual sera, the final dilution of all the sera in the pools was chosen to be either 1:40 or 1:20. Hyperimmune sera of the Coxsackie types giving "unreliable" CF reactions with the prototype antigens (1) were however, always employed in a dilution 1:16.

In Tables 5 and 6 are presented results obtained with 5 CF antigens when these higher serum dilutions were employed. It will be seen that even here slight non specific reactions were now and then encountered, but otherwise full agreement was obtained between typing by CF tests and by neutralization tests in newborn mice.

It was investigated whether employing the rather poor antigen No 6845 in a volume of 0.2 ml instead of the usual 0.1 ml would improve the results. It was found that 0.2 ml gave slightly stronger reactions with pool I than did 0.1 ml, but the titrations of complement showed

that the application of a double amount of antigen also resulted in an appreciable increase of its anticomplementary effect

Table 7 presents a summary of the CF typing of all the 23 stool strains of Coxsackie virus included in the study. In 21 out of the 23 samples examined agreement between the neutralization tests and the CF tests was obtained when second mouse passage material was used for CF antigen preparation.

In the two instances where second mouse passage did not provide a sufficiently potent CF antigen the experiments were repeated using antigens prepared from third mouse passage material. Typing by the CF method was now successful with both strains, one A 16 strain and one A 1 strain respectively.

### DISCUSSION

In a preceding study (1) it was found that satisfactory CF antigens could be prepared from the prototype strains of 18 out of 24 prototype Coxsackie A strains while 6 prototype antigens (types 11, 13, 17, 18, 19 and 21) gave negative or unreproducible results.

The present experiments have shown that the CF technique could be employed as routine method for typing of 23 Coxsackie A virus strains freshly isolated in newborn mice which represented all the Coxsackie A virus isolated—other than type 9<sup>2</sup>—available in the laboratory at the time of the experiments. Satisfactory CF antigens were thus prepared from second mouse passage of 21 out of the 23 Coxsackie A strains. One A 1 strain and one A 16 strain required a third passage in mice before yielding a good antigen. None of the 23 strains belonged to the difficult 6 Coxsackie A types listed in the beginning of this chapter.

Since Coxsackie B viruses do not usually yield satisfactory CF antigens when mouse tissue material is employed for the preparation (1) tissue culture antigen is preferable for CF typing of the Coxsackie B types and no further attempts have been made in our laboratory with mouse tissue CF antigens of the Coxsackie B strains.

From earlier experiments it was concluded that only mouse tissue suspensions with a reasonably high infectious titer would consistently yield reliable CF antigens (1). This observation has been confirmed by the results of infectivity titrations of the present CF antigens. It will thus be seen from the tables that CF antigens prepared from high titer Coxsackie A virus strains showed good CF potency and were all type 1.

When third mouse passage antigen showed low or no CF potency. When third mouse passage of these virus strains was used for antigen preparation they both gave reliable although weak

<sup>2</sup> For CF typing of Coxsackie A 9 virus strains tissue culture antigens have rarely been employed in this laboratory (4).

fixation with the homologous sera. The infectious titers of these 3' passage antigens were ca  $10^{-5.9}$  and  $10^{-5.6}$ , respectively.

Whether the infected material from the suckling mice will yield a satisfactory CF antigen can to some extent be predicted from the ratio of mice succumbing to infection and from the character and degree of paralysis, and from the incubation time. It has been our experience that the majority of the satisfactory antigens was obtained when definite signs of illness was observed in most of the mice on the second or third day after inoculation.

The bacterial content in an inoculum may occasionally give signs of illness in the mice resembling those produced by Coxsackie viruses. In such instances ether treatment of the inoculum and addition of adequate antibiotics have proved useful.

The exact amount of complement actually present in the various test tubes is one of the important factors in CF typing experiments. This is illustrated by the difference in fixation grades observed in the first and second type experiments with the antigens Nos. 5913 and 5913-3 (Tables 3 and 4) and with antigen No. 7119 (Tables 5 and 6). When weak antigens are employed it would in some instances undoubtedly be useful if several dilutions of complement were included in the same experiment (5).

In the present study slight non-specific reactions were occasionally encountered when the sera were used in pools even in the dilutions 1/20 and 1/40. Since these reactions never were stronger than + to 1 reactions, they were, however, not difficult to distinguish from specific reactions. The non-specific reactions were found more often when the sera had been stored at  $+4^{\circ}\text{C}$  instead of at  $-20^{\circ}\text{C}$  before use in CF tests. To avoid non-specific reactions it seems generally advisable to include the individual sera in the pools in a dilution four to eight times lower than the CF antibody titer of the serum, even if this will make the preparation of the serum pools slightly more laborious (1). However, in certain instances, particularly when antigens expected to be of low potency are to be tested, the use of more concentrated sera may still be preferable.

The number of freshly isolated strains examined in this study has been relatively small. However, the data from this and preceding studies (1, 2) show that although it may be necessary to resort to typing by neutralization tests in newborn mice of certain Coxsackie A strains, the described CF method may be expected to be a valuable tool in routine typing of the majority of freshly isolated Coxsackie A virus strains.

#### SUMMARY

In the present study Coxsackie A virus strains isolated from patients by inoculation of newborn mice have been typed by the complement fixation technique.



The CF antigens were prepared from mouse torso tissue employing treatment with trichlorotrifluoroethane ("Freon 112"), and guinea pig serum was subsequently added (1, 2)

The 23 Cocksackie A virus strains examined comprised six A 1, one A 2, two A 4, five A 5, three A 8, one A 10, and five A 16 strains. Satisfactory CF antigens were prepared from second mouse passage of 21 out of 23 of the freshly isolated strains. One Cocksackie A 1 strain and one Cocksackie A 16 strain could be typed after one additional mouse passage.

Various technical problems concerning the practical use of the CF method for routine typing of Cocksackie A virus strains freshly isolated in newborn mice are discussed.

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## IMMUNOCHEMICAL STUDIES ON POLYSACCHARIDES FROM STAPHYLOCOCCUS EPIDERMIDIS

### 1 Isolation and Chemical Characterization

By

NORVALD LOSVIGARD and PER OEDING

Received 4:163

On the basis of the precipitation test *Julianelle & Wieghard* (26) have divided staphylococci into pathogenic type A and nonpathogenic type B strains containing the corresponding carbohydrates A and B. Later *Thompson & Khorazo* (36) described three new groups, C, BC, and O, following precipitation of nonpathogenic strains. *Staph aureus* strains lacking carbohydrate A were placed into another group by *Cowan* (6). Carbohydrates A and B have to some extent been characterized chemically by *Wieghard & Julianelle* (37). Significant differences in optical rotation, fermentable and nonfermentable sugars were found. Detailed chemical and serological examinations of polysaccharide A from two *Staph aureus* strains have been reported by *Haukenes* (14-20). Polysaccharide A was found to be serologically identical and chemically very similar to teichoic acid from the wall of *Staph aureus* (21).

To our knowledge there have been no further reports on the chemical composition of the group-specific polysaccharides present in *Staph epidermidis*.

### MATERIALS AND METHODS

**Strains** Four *Staph epidermidis* strains were used in the present experiments. Strain 1254 was isolated from the nose, 1268 from pus and 1622 and 3519 from urine. All the strains were negative on coagulase, mannitol, hyaluronidase, phosphatase and esterase test. The strains used by *Julianelle & Wieghard* (26) and *Thompson & Khorazo* (36) were not available.

**Growth and harvesting** Eighteen hour cultures of the strains grown on nutrient agar in Petri dishes of 14 cm diameter were harvested by scraping with a glass rod.

**Disruption of cells** was obtained in a Hughes bacteria press at  $-25^{\circ}\text{C}$  (25).

**Extraction** The extraction procedure described by *Haukenes et al* (22) was adopted with some modifications. The disrupted cells were suspended in distilled water, stirred and centrifuged. The supernatant was adjusted to pH 4.2 with 0.1 N HCl and the precipitate removed. After raising the pH to 6.9 with 0.1 N NaOH the supernatant was used for further extraction of the cells. This process was repeated 10 times. On the two last occasions the microbes were resuspended in distilled water and the pH brought to 4.2 before centrifugation. Extraction and centrifugation were performed in the cold except for strain 3519 which was extracted in an incubator at  $37^{\circ}\text{C}$ .

Ion exchange columns were used for further purification of the polysaccharide material

Diethylaminoethyl (DEAE) cellulose 100-230 mesh was prepared as described by Peterson & Sober (31). The procedure for running the column was principally that described by Haukenes (14). The system was arranged to give a continuous increase of the KCl gradient from 0 to 0.4 M.

De-Acidite FF columns. The resin (The Permutit Co. London) of 100-200 mesh and 3.5 per cent cross linkage was suspended in 80 per cent formic acid and supplied to the column. More 80 per cent formic acid was run through then water 3.25 M ammonium formate of pH 5.2 and finally water. Both stepwise and continuous increase in the ammonium formate gradient up to a molarity of 3.0 were tried.

Amberlite IR 120. The resin (Rohm & Haas Co. Philadelphia) 100-230 mesh 8 per cent cross linkage was employed to fractionate sugars, amino sugars and amino acids in the hydrolysate of the polysaccharide material purified on the foregoing columns. Stepwise elution with 0.33 N HCl (7), 1.5 N HCl (2.5 N HCl) and 4 N HCl (35) was used.

Dialysis was carried out in cellophane tubes (Kalle & Co. Wiesbaden).

Sephadex G 25 and Sephadex G 50 (Pharmacia Uppsala) columns were used for gel filtration (14).

Hydrolysis was performed in 3 N HCl for 3 hours at 100°C in glass stoppered tubes.

Paper chromatography. The fractions from the Amberlite IR 120 columns were pooled according to the preliminary tests for sugars, amino sugars and amino acids. The batches were evaporated to dryness under reduced pressure. Descending chromatograms on Whatman No 1 paper as supplied were run in the following systems:

A. Phenol water (4:1 w/v) (4). The phenol was purified by distillation with cent ammonia on the bottom of

F. Methanol water 10 N HCl pyridine (80:17.5:2.5:10) (32)

#### Spray reagents

a. Ninhydrin 0.1 per cent in water saturated n-butanol (4) or in acetone (24) amino sugars on paper chro

Paper etc.

#### Analytical methods

Aminosamines were determined by the method of Rondle & Morgan (33).

For carbohydrates the quantitative Molisch test described by Dische (8) was employed.

Relative viscosity was determined in an Ostwald viscometer (16) on aqueous solutions of materials at a concentration of 5 mg/ml.

Specific rotation was measured in a Zeiss Circle Polarimeter (16).

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**Disruption of cells** was obtained in a Hughes bacteria press at  $-25^{\circ}\text{C}$  (22).

**Extraction** After washing the cells with distilled water, they were suspended in distilled water and stirred and centrifuged.

After raising the pH to 8.2 with 0.1 N NaOH, the supernatant was used for further extraction of the cells. This process was repeated 10 times. On the two last occasions the microbes were resuspended in distilled water and the pH brought to 4.2 before centrifugation. Extraction and centrifugation were performed in the cold except for strain 3519 which was extracted in an incubator at  $37^{\circ}\text{C}$ .

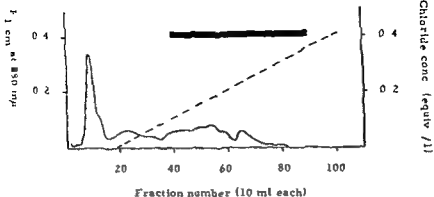


Fig 1

Chromatography of polysaccharide 1254 on DFAE cellulose

- $E_{1\text{ cm}}$  at 280  $m\mu$   
 --- Chloride concentration  
 ■ Major part of polysaccharide material

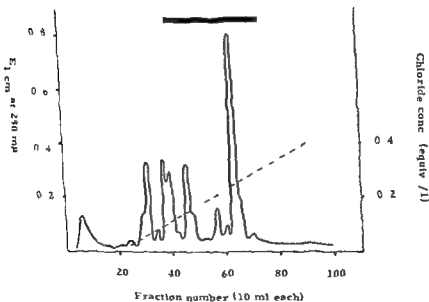


Fig 2

Chromatography of polysaccharide 1622 on DFAF cellulose

- $E_{1\text{ cm}}$  at 280  $m\mu$   
 --- Chloride concentration  
 ■ Major part of polysaccharide material

Deoxypentoses and pentoses were examined by Dische's diphenylamine test (9) and Bial's test (2) respectively

The qualitative Molisch test for carbohydrates and the qualitative biuret test for proteins were carried out as described in (22)

Precipitation by ring test was performed by the method used by Haukenes *et al* (22)

Agar precipitation, as used by Haukenes & Oeding (23), was used for the examination of polysaccharides during the purification steps

The immune sera used in the precipitation tests were produced by intravenous injections of formalin-killed bacteria as described by Oeding (28)

## RESULTS

It was observed that if the suspensions of strains 1254, 1268, and 1622 were placed in an icebox for 2-4 weeks after the first series of extractions had been completed, large amounts of serologically active material were released. In strains 1268 and 1622 this material formed the major part of the total active material obtained. Further extractions gave only negligible amounts. The first extraction series of strain 3519 included also repeated extraction at 37° C for 2-3 days. In contrast to the above observation strain 3519 appeared to be nearly emptied of active material during the first extraction period, as further extraction in an icebox, followed by extraction at 37° C gave but little active material. This is in accordance with the observation by Haukenes (20) that polysaccharide A is released from the cell wall of *Staph aureus* by a lytic enzyme.

The polysaccharides showed rapid migration on agar precipitation. On dialysis serologically active material passed through the cellophane membrane very slowly. The loss of material on dialysis over night, as calculated from ring test precipitation, was about 0.5 per cent.

The yield of purified polysaccharides varied from about 5 to about 12 mg per g wet weight of bacteria.

### Ion Exchange Chromatography

Polysaccharide 1268 produced a curve similar to that of polysaccharide 1254 (Fig. 1), whereas the 3519 preparation behaved like 1622 (Fig. 2) in the cellulose column. Some ultraviolet light absorbing material goes straight through the columns. Serologically active material is mainly excluded in the region 0.1-0.35 M KCl, as examined by the ring test. The De-Acidite FF columns also excluded ultraviolet light absorbing material at the beginning of the run, whereas active material was liberated from just below 1 M to 3 M ammonium formate. Ultraviolet light absorption, obviously not associated with the serologically active material, also occurs in the region of the active material.

Ultraviolet light absorbing fractions were removed as far as possible by ion exchange chromatography without losing too much of the active material. During the purification steps the material showed increasing ring test titre and diminishing ultraviolet light absorption. The

sence of glucosamine. Amino sugar test was carried out on the 1268 hydrolysate fractions, measuring the extinction at  $530\text{ m}\mu$ , immediately and after  $1\frac{1}{2}$  hours, and at  $505\text{ m}\mu$  after 24 hours. The last 3 positive fractions of a total of 10, showed markedly higher extinction values at  $505\text{ m}\mu$  than at  $530\text{ m}\mu$ , thus behaving like muramic acid (7). Corresponding examinations of the other polysaccharide hydrolysates were not performed. The amino acids, as pointed out by paper chromatography, were excluded in the order reported by Stein & Moore (35).

### *Paper Chromatography*

While system A indicated the presence of lysine in the polysaccharide preparations from all the strains, there were some difficulties in detecting lysine in system B as the paper was discoloured in the appropriate region. The material was therefore run in system E and sprayed with 0.1 per cent ninhydrin in acetone, and the characteristic purple colour, which changed to brown, developed. Also the distance travelled was in good agreement with that for the lysine reference. This chromatogram also excluded the presence of  $\alpha$ -diaminopimelic acid, which, besides, has not been found in staphylococcal cell walls (34). One more chromatogram was run in system E, the spots eluted and run anew in system B and lysine was found to be present. Glutamic acid, glycine, alanine and serine in addition to lysine, were found in the polysaccharide preparations from all the strains.

The pooled fractions containing amino sugars gave 2 well separated spots with the Fison-Morgan reagents both in system A and B. Ninhydrin oxidized (12) polysaccharide hydrolysates from all the strains gave spots corresponding to arabinose in system A and C, showing the major amino sugar component to be glucosamine. The  $R_F$  values of the other component were in good agreement with those for muramic acid found by Crumpton (7).

The Molisch positive materials from the Amberlite IR-120 columns were run in system A and spots with  $R_F$  0.35-0.37 were eluted and used for running in system C and for electrophoresis. Glucose seemed to be present in every one of the preparations.

Samples of 1-2 mg polysaccharide material were hydrolyzed, evaporated to dryness and examined for sugar alcohols in system B. As references were used glycerol and hydrolyzed samples from a ribitol tetrachloro acid and a glycerol tetrachloro acid. The tetrachloro acids were kindly furnished by Professor J. Baddiley, Newcastle. Glycerol was found in the strain 1254 and 1268 material whereas ribitol and anhydrosorbitol were found in the preparations from 1622 and 3519.

Small amounts of Fison-Morgan reacting material, assumed to be unhydrolyzed residues, were detected on paper chromatograms. These substances were eluted prior to the amino sugars on the Amberlite IR-120 column.

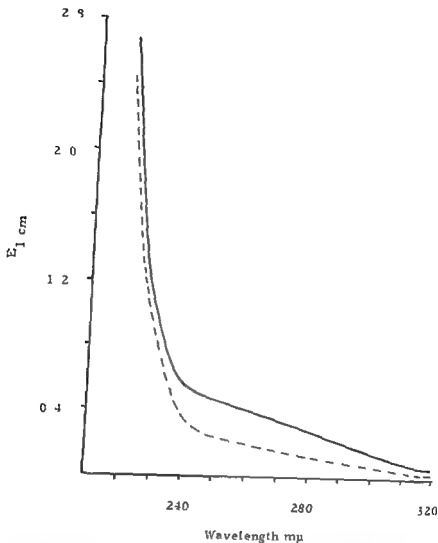


Fig 3

Ultraviolet absorption spectra of polysaccharide preparations from strains 1254 ( - ) and 1622 ( — ) both in a concentration of 5 mg/ml in aqueous solution

ultraviolet absorption spectra of polysaccharides 1254 and 1622 are shown in Fig 3

Molisch positive material from 25 mg hydrolyzed polysaccharide samples went straight through the Amberlite IR-120 columns, the test being strongly positive on the samples from strains 1254 and 1268, weakly positive on those from strains 1622 and 3519. Amino sugars started to go out at a volume of about 70 ml and were completely excluded at about 100 ml on elution with 0.33 N HCl. The volume from the start to the fraction which contained the greatest amount of amino sugars was compared with the corresponding volumes for glucosamine and galactosamine references. For glucosamine the volume was 86 ml, for galactosamine 92 ml. For the amino sugars from the 4 polysaccharides the volumes varied from 85 to 87 ml, thus indicating the pre-



### Analyses of Polysaccharides

Table 2 comprises all the data obtained from the quantitative as well as the qualitative analyses of the polysaccharide preparations. The nitrogen content is low in each of the polysaccharides, whereas the reducing sugars, calculated as glucose, and the phosphorus content are high. Inorganic phosphorus was not present. The hexosamine content of polysaccharides 1622 and 3519 is markedly higher than that of the polysaccharides 1254 and 1268. However, the most significant differences between polysaccharides 1254 and 1268, on the one hand, and those of 1622 and 3519 on the other are the neutral sugar content, the specific rotation and the presence of glycerol in the former group and ribitol in the latter.

The amino acids alanine, glycine, glutamic acid, lysine, and serine were demonstrated in each of the polysaccharides. Visually glycine and alanine seemed to be present in large amounts, whereas the content of serine was small.

### DISCUSSION

Crude polysaccharide 1622 (and 3519) seems to be more contaminated with ultraviolet light absorbing materials than crude polysaccharide 1254 (and 1268), as the former, in contrast to the latter, produces several distinct peaks (Fig. 1 and 2).

The finding that the activity increased with decreasing ultraviolet light absorption during purification of the materials indicates that absorption is not associated with the serologically active material. There is however still some ultraviolet light absorbing material present in the purified polysaccharides (Fig. 3). None of them produced the nucleic acid peak at 260  $m\mu$ , nor the protein peak at 280  $m\mu$ , which is in accordance with the negative Bial and Dische tests (Table 2), the low nitrogen content found on analysis, and the lack of aromatic amino acids in acid hydrolysates. It is believed that re-chromatography on columns together with an even more drastic removal of ultraviolet light absorbing fractions would have rendered the polysaccharides almost free of ultraviolet light absorbing material. One reservation has to be made. As will be reported in a forthcoming article, the preparations may be mixtures of more antigens and the weaker ones may possess ultraviolet light absorbing ability.

All the polysaccharides were excluded on the Sephadex G 50 column, suggesting that the molecular weights were above 10,000 to 100,000. According to the low relative viscosity, the partial dialyzability and the rapid migration in agar the molecular weights can, however, not be very high. These statements are valid for polysaccharide A too, as reported by Haukenes (16).

The difference in carbohydrate content of polysaccharides 1622 and 3519 is compared to polysaccharides 1254 and 1268 must be due to a

## Electrophoresis

The paper chromatograms indicated that glucose was present. Verification of this by electrophoresis was only partly successful.

Only traces of a sugar, that behaved as glucose, were found in polysaccharide 3519. Polysaccharide 1622 contained a sugar that behaved like glucose at pH 9.2 and like galactose at pH 8.6. The converse was the case for the sugar from polysaccharide 1254. These findings have to be checked with new portions of material. The results obtained with the sugar from polysaccharide 1268 are listed in Table 1. The sugar is most probably glucose.

TABLE 1

*Mobilities on Paper Electrophoresis of the Sugar from Polysaccharide 1268 and Reference Sugars in Borate at various pH Values, on Whatman No 1 Paper*

Sugar	Distance from origin cm		
	pH 9.2	pH 8.6	pH 7.0
Polysaccharide 1268 sugar	11.4	11.5	9.8
Mannose	7.2	5.7	
Arabinose	11.5	12.3	
Glucose	11.5	11.3	9.5
Galactose	10.8	9.9	10.0

TABLE 2  
*Analytical Data*

	Polysaccharide from <i>Staph. epidermidis</i> strain			
	1254	1268	1622	3519
Nitrogen, per cent	2.34	3.43	2.93	3.20
Phosphorus, per cent	7.72	6.98	6.76	7.20
Reducing sugars (as glucose), per cent	42.16	46.63	40.80	40.80
Hexosamines (as glucosamine), per cent	13.69	16.45	23.34	24.93
Carbohydrates (as glucose), per cent	17.82	17.42	2.06	0.75
Relative viscosity	1.12	1.12	1.20	1.23
Specific rotation ( $\alpha$ ) <sub>D</sub> 22°	47.8°	49.8°	11.0°	47°
Biuret *	±	+	—	—
Bial *	—	—	—	—
Dische *	—	—	—	—
Millon *	—	—	—	—
Glucosamine	+	+	+	+
Muramic acid	+	+	+	+
Glucose †	(+)	+	(+)	±
Glutamic acid	+	+	+	+
Serine	+	+	+	+
Glycine	+	+	+	+
Alanine	+	+	+	+
Lysine	—	—	+	+
Ribitol	—	—	+	+
Anhydrosorbitol	+	+	—	—
Glycerol	+	+	—	—

\* Concentration of polysaccharide 5 mg/ml

† Brackets denote that the sugar found is assumed to be glucose



difference in the amount of glucose, as this sugar was the only one found on qualitative estimation. It seems reasonable to assume that the weak reactions for glucose found in polysaccharides 3519 and 1622 are due to contaminants.

The reducing capacities found are greater than those that can be calculated from the hexosamine and carbohydrate content. Interaction of amino acids may render the reducing value too high. On the other hand, the hexosamine content, determined as glucosamine, is likely to be too low as the muramic acid present, according to Crumpton (7), only produces about 27 per cent of the colour intensity of an equal weight of glucosamine at 530 m $\mu$ . Furthermore, with the conditions of hydrolysis employed, there were still small amounts of unhydrolyzed, Elson-Morgan reacting material left as pointed out by paper chromatography.

With milder conditions of hydrolysis, our amino sugars, estimated on paper chromatograms, probably would have appeared to be N-acetyl derivatives since most naturally occurring amino sugars contain N-acetyl groups.

The amino acids alanine, glycine, glutamic acid, lysine, and serine, as demonstrated in each of our polysaccharides, are regularly found in the walls of staphylococci (34), and constitute, together with glucosamine and muramic acid, the mucopeptide of the wall.

According to Haukenes (17, 20) polysaccharide A constitutes a part of the cell wall of *Staph aureus* and is composed of two structural components, a mucopeptide and a ribitol teichoic acid. The present examinations indicate that the purified polysaccharide material isolated from *Staph epidermidis* strains 1254 and 1268 contains a glycerol teichoic acid-mucopptide, whereas the polysaccharide from *Staph epidermidis* strains 1622 and 3519 contains a ribitol teichoic acid-mucopptide.

The analytical data from polysaccharides 1622 and 3519 are very similar to those found by Haukenes (16-20) in polysaccharide A. The difference in optical rotation, a dextrorotation of +5 to 11° in polysaccharides 1622 and 3519 compared to a laevorotation of -11.5° in polysaccharide A (16), may, however, be of importance. The small amounts of glucose found in polysaccharides 1622 and 3519 may influence the optical rotation. There is also a possibility that the difference is due to the glycosidic linkages present, e.g. that polysaccharides 1622 and 3519 contain more  $\alpha$ -glycosidic linkages than polysaccharide A. In the subsequent paper on the antigenic properties of polysaccharide 1622 and 3519 it will be shown that this polysaccharide also has the  $\alpha$  glycosidic linkages, which determine the serologic specificity of polysaccharide A (21).

The values obtained for optical rotation, carbohydrates, and fermentable sugars in our polysaccharides 1254 and 1268 are in good agreement with the values reported by Wieghard & Julienne (37) for carbohydrate B.

## IMMUNOCHEMICAL STUDIES ON POLYSACCHARIDES FROM STAPHYLOCOCCUS EPIDERMIDIS

### 2 Antigenic Properties

By

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Received 4163

The information on the antigenic properties of *Staphylococcus aureus* has recently been reviewed by Oeding (10). Little is, however, known of the antigenic properties of coagulase negative staphylococci.

Julianelle & Wiegand (6) reported that pathogenic and nonpathogenic staphylococci could be differentiated by means of specific carbohydrate precipitinogens which they called A and B respectively. Thompson & Khorazo (13) described additional types of nonpathogenic staphylococci, C, BC, and O. These authors and Verwey (14) found type A strains to be mannitol-,  $\alpha$ -toxin-, and coagulase positive, whereas B, C, BC, and O strains were negative with these tests. Unfortunately these type strains seem to have been lost and a comparison with carbohydrates A and B has to be based mainly on chemical characterization. (15) Haukenes (2) demonstrated that of 100 *Staph. aureus* strains 97 produced the polysaccharide A line on agar precipitation, whereas all the 62 *Staph. epidermidis* strains examined by the same technique, failed to give this line. Ten of the latter strains were also examined by a more sensitive technique. He was then able to demonstrate the polysaccharide A line in three of these strains.

In a previous paper (7) the chemical characteristics of two types of purified polysaccharide from strains of *Staph. epidermidis* have been described. One type was thought to be a glycerol teichoic acid mucopolysaccharide and showed definite similarity to Julianelle & Wiegand's (6) carbohydrate B, whereas the other, thought to be a ribitol teichoic acid mucopolysaccharide, seemed to be structurally similar to polysaccharide A of *Staph. aureus*. In the present paper these polysaccharides and a number of other *Staph. epidermidis* strains have been examined serologically.

### MATERIALS AND METHODS

**Strains.** One hundred and twenty-five coagulase negative *Staph. epidermidis* strains from our routine material including the strains 1254, 1263, 1622 and 3519 chemically characterized in (7), were examined. Four pathogenic *Staph. epidermidis*

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## IMMUNOCHEMICAL STUDIES ON POLYSACCHARIDES FROM STAPHYLOCOCCUS EPIDERMIDIS

### 2 Antigenic Properties

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In a previous paper (7) the chemical characteristics of two types of purified polysaccharide from strains of *Staph. epidermidis* have been described. One type was thought to be a glycerol teichoic acid mucopolysaccharide.

In the present paper these polysaccharides and a number of other *Staph. epidermidis* strains have been examined serologically.

### MATERIALS AND METHODS

**Strains.** One hundred and twenty five coagulase negative *Staph. epidermidis* strains from our routine material including the strains 1254, 1258, 1622 and 3519 chemically characterized in (7) were examined. Four pathogenic *Staph. epidermidis*

strains were supplied by Maclean Smith Iowa City (12) The *Staph aureus* type strain 1503 (8) was used for comparison

Purified polysaccharide material was prepared from strains 1254 1268, 1622 3519, and 1503 (7)

Immune sera against the 5 strains above were produced by intravenous injections of formalin-killed bacteria by Oeding's (9) method

Absorption was carried out as described by Haukenes (2)

Precipitation using the ring test was performed by the method used by Haukenes et al (4)

**Agar diffusion technique** The agar medium and the plates were prepared as described in (5) The thickness of the agar gel was about 2 mm One central and 6 peripheral, circular basins of 12 mm diameter were cut, the distance between them being 10 mm The technique used for the examination of the purified polysaccharides has been described in (5)

A special technique which offered good opportunities for observing the characteristic polysaccharide lines was used for the examination of the routine strains All strains were examined against antisera 1254 3519, and 1503 using polysaccharides 1254 3519, and 1503 as references Alternate peripheral basins were filled with glucose broth and inoculated with the strains to be tested After incubation at 37° C for 24 hours the central basin was filled with antiserum Six hours later the polysaccharide, usually in a concentration of 0.1 mg/ml was put into the remaining peripheral basins

Though it produces the same polysaccharide lines antiserum 3519 was preferred to antiserum 1622 as the latter was a rather weak antiserum Antisera 1254 and 1268 possessed the same antibodies The former was selected as it appeared to be the stronger one, producing more distinct lines

## RESULTS

### Ring Test Precipitation

According to the strong cross precipitation (Table 1) there must be a close relationship between polysaccharides 1254 and 1268, and also between those of 1622, 3519, and 1503 This is confirmed by the results of agar gel precipitation (see below) Some cross-precipitation also takes place between polysaccharides of the two groups, the titres for polysaccharide 1254 against antiserum 1503 and polysaccharide 1622 against antiserum 1268 being rather high

TABLE 1  
Ring Test Precipitation with Staphylococcal Polysaccharide Materials

Antiserum	1254	1268	1622	3519	1503
1254	1 4 000	1 3 000	1 100	1 100	1 100
1268	1 4 000	1 3 000	1 100(0)	1 100	1 100
1622	1 10	1 10	1 2 000	1 3 000	1 1 000
3519	1 100	1 100	1 1 000	1 3 000	1 2 000
1503	1 100(0)	1 100	1 2 000	1 3 000	1 5 000

The titre indicates the highest dilution of antigen (mg/ml) giving precipitation  
Antiserum undiluted

According to the absorption experiments, polysaccharides 1622 and 3519 seem to have one rather strong antigen which is not shared by the other polysaccharides This is apparently the case also with polysaccharides 1254 and 1268 Polysaccharide A (1503), however, does



not seem to have any antigenic specificity which is not also found in the 1622 and 3519 polysaccharides. As the sera used for these experiments are produced against the whole organisms, the relationship between the polysaccharide materials is very difficult to evaluate. Specific sera have not been obtained as the polysaccharides were non antigenic in rabbits.

#### Agar Gel Precipitation

The behaviour of the polysaccharide preparations on agar gel diffusion is shown in Fig 1-3. Each of the preparations produces more than one line against the homologous serum. Only those lines which seem to be definitely established are illustrated in the figures. In accordance with the precipitation reactions (Table 1), polysaccharides 1254 and 1268 behave identically on agar diffusion also, as both the observed lines give a reaction of identity. Polysaccharide 1622 has four lines in common with polysaccharide 3519 against antiserum 3519, two of which are also produced by polysaccharide 1503 (Fig 3). The line designated I is weak and not always easy to demonstrate. A strong antiserum is required as the antigenic part of the system migrates rapidly into the antibody basin. Line II, which is also demonstrated against antiserum 1503 (Fig 2) is the polysaccharide A line. Polysaccharide A is apparently responsible for the strong cross precipitation observed in the ring test between strain 1503 and strains 1622 and 3519. Lines III and IV (Fig 3) are regarded as the principal lines of strains 1622 and 3519.

After absorption of sera 1622 and 3519 with polysaccharide 1268 and polysaccharide 1503 these sera still produced lines III and IV against polysaccharide 1622, whereas polysaccharide 1503 gave no line. As polysaccharide 1268 had not been shown to give a polysaccharide A line (II) its ability to remove the corresponding antibody seemed

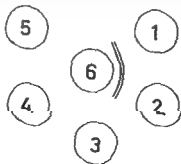


Fig 1

Schematic drawing of  
Polysaccharide  
1 2 3 and 5 re  
Antiserum 1254

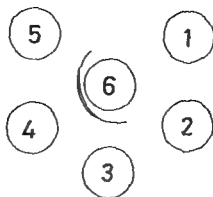


Fig 2

Schematic drawing of lines produced against antiserum 1503 on agar gel diffusion Polysaccharide from *Staph epidermidis* strains 1268 1254, 1622 and 3519 in wells 1, 2, 3, and 5 respectively Polysaccharide from *Staph aureus* strain 1503 in well 4 Antiserum 1503, undiluted, in well 6 All polysaccharides in 0.1 mg/ml concentration

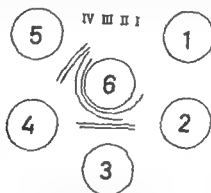


Fig 3

Schematic drawing of lines produced against antiserum 3519 on agar gel diffusion Polysaccharide from *Staph epidermidis* strains 1268 1254, 1622 and 3519 in wells 1, 2, 3 and 5 respectively Polysaccharide from *Staph aureus* strain 1503 in well 4 Antiserum 3519 undiluted in well 6 All polysaccharides in 0.1 mg/ml concentration

strange, although analogous results had been obtained on ring test precipitation. The following experiment may also indicate that polysaccharides 1254 and 1268 have small amounts of polysaccharide A or that they have part of a structure in common with polysaccharide A. Antisera 1622 and 3519 absorbed with excess polysaccharide 1268, produced a line which gave a probable reaction of identity with polysaccharide A against antiserum 1503. This should be compared with the findings of Haukenes (2) mentioned in the introduction. It is also possible that line I is present in polysaccharides 1254 and 1268. These possibilities await further investigation.

In Table 2 are listed the results of agar gel precipitation with 125 strains of *Staph epidermidis* selected from our routine material. All strains were tested against reference systems 1254, 3519, and 1503. The strains were classified according to the lines showing reactions of

identity with the reference polysaccharide lines (Fig 1-3), although line I was excluded. Thus, the designation group A means that a strain has the polysaccharide A line exclusively, and group B that a strain has the double line found in polysaccharides 1254 and 1268. The double line characteristic of polysaccharides 1622 and 3519 has been designated C, and because these polysaccharides also have an A line, the group designation is AC.

TABLE 2

Examination of Routine Strains of *Staph. epidermidis* by Agar Gel Diffusion

No of strains	Origin	Group according to polysaccharide lines					
		A	B	AB	AC	ABC	0
38	Nose	2	27	0	6	1	0
6	Throat	1	3	0	0	1	1
14	Pus	0	7	0	4	0	3
9	Blood	0	5	0	1	1	2
9	Others	0	5	0	0	0	4
Sum 74		3	47	0	11	3	10
30*	Urine	1	13	0	8	2	6
21†	Urine	0	3	0	16	1	1
Sum 51		1	16	0	24	3	7
Total 125		4	63	0	35	6	17

Typing was based on a reaction of identity with the reference polysaccharide group III strains produced no line.

\* Strains with no or a dubious relationship to infection.

† Strains with possible relationship to infection.

The strains were selected with the intention of comparing the precipitinogens present in strains which had almost certainly no pathogenic significance, with strains which were considered to be the causative agents of the infections from which they were isolated. If a sample of pus or urine showed Gram positive cocci in heaps and leucocytes microscopically and gave a pure culture of *Staph. epidermidis*, it was considered possible that the strain was responsible for the infection.

Table 2 shows a certain correlation between the polysaccharide group and the origin of the material. The most striking difference is noticed between the nose strains and the strains considered to be responsible for urinary infections. Seventy-five per cent of the nose strains belong to group B whereas 76 per cent of the urinary strains, group 2, are AC strains. The 1 strain registered under group A may have small amounts of C antigen and thus belong to group AC.

Four strains of *Staph. epidermidis* which have been reported by Smith *et al.* to have caused septicemia (12), were also examined. All these strains were of polysaccharide group B.

## DISCUSSION

A previous article (7) dealt with the chemical characterization of the polysaccharides. Polysaccharides 1254 and 1268 were found to belong to one group, whereas polysaccharides 1622 and 3519 belonged to another. This is supported by their antigenic properties.

Polysaccharides 1622 and 3519 seem structurally to be very similar to polysaccharide A, the only significant difference being dextrorotation in the former and laevorotation in the latter (7). The present investigations demonstrate at least two serological specificities of polysaccharides 1622 and 3519, one being the same as that of polysaccharide A. The other specificity may also be found in the ribitol teichoic acid structure, and as  $\beta$ -glycosidic linkages are responsible for the serological specificity of *Staph aureus* teichoic acid (3), the possibility must be considered that  $\alpha$ -linkages are responsible for the other specificity. The serological activity may also be ascribable to the mucopolypeptide moiety.

The polysaccharide of *Staph epidermidis* strains 1254 and 1268 is probably a glucose-glucosamine glycerol teichoic acid-mucopolypeptide A. A wall teichoic acid of similar composition was isolated by Ghuyssen (1) from a strain of *B. megaterium*. The strong specific serologic reactivity of our polysaccharide may be ascribable to the glucose linkage, whereas the weak cross-reaction with polysaccharide A and polysaccharides 1622 and 3519, can be explained by the presence of  $\beta$ -glucosamine linkages.

The significance of the line designated I (Fig. 3) is at the moment rather obscure, but the antigen involved seems to be widely distributed among the strains examined. The question arises whether this antigen may play a part in some of the cross reactions observed between strains from different groups.

Due to the chemical composition (7) and antigenic properties of their polysaccharides, the strains 1254 and 1268 are regarded as group B strains, i.e. type B strains of Julianelle & Wughard (6). It may be reasonable to use the designation AC for the serologic reactivities demonstrated in polysaccharides 1622 and 3519, until the chemical nature of the active groups has been definitely determined.

The examination of 125 routine strains of *Staph epidermidis* revealed a certain correlation between polysaccharide group and origin of the strains. Although there may be other reasons for this distribution, the findings might indicate that certain types of *Staph epidermidis* are associated with the place of growth. It is also possible that strains capable of causing infections have different polysaccharide antigens than purely saprophytic strains. It has been shown in the present investigation that one group of *Staph epidermidis* strains possess polysaccharide A, which is otherwise characteristic of *Staph aureus*. However, it seems that in these strains polysaccharide A is a minor antigen, and

always (or nearly always) associated with another, major antigen (C) *Staph aureus* strains always seem to have polysaccharide A. One might therefore think that polysaccharide A is associated with the ability to cause infection. If so, pathogenic strains of *Staph epidermidis* should have polysaccharide A as a minor antigen. The results shown in Table 2 seem to support this theory as 76 per cent of the group of urinary strains with possible relation to infection are of polysaccharide group AC. In contrast the majority of the nose strains, which should be expected to be saprophytic, belong to group B. However, 4 strains reported by Smith et al (12) to have been responsible for cases of septicemia, were shown by us to be polysaccharide B strains. Recently Pereira (11) reported that strains of *Staph epidermidis* responsible for urinary infections, all had a certain agglutinin, but the chemical nature of this is unknown.

It is accepted that *Staph epidermidis* may be able under certain circumstances to cause infections in man. Therefore, it would be of interest to extend our very poor knowledge of the antigens of these organisms.

#### SUMMARY

Purified polysaccharide preparations from four *Staph epidermidis* strains, examined by ring test and agar gel precipitation were found to comprise two serological groups, B and AC. This is in accordance with the chemical data presented in a previous article. There was strong cross precipitation between polysaccharide A of *Staph aureus* (group A) and the AC polysaccharide, due to the presence of polysaccharide A in *Staph epidermidis* group AC strains. The purified *Staph epidermidis* polysaccharides thus have more than one serological specificity.

The AC polysaccharide is thought to contain glucosamine ribitol teichoic acid with  $\beta$  glycosidic linkages that react serologically with polysaccharide A and another grouping determining the specificity of the substance. The B polysaccharide probably contains a glycerol teichoic acid with glucose linkages which may determine the strong specific reaction and  $\beta$ -glucosaminic linkages which may give cross reactions.

A number of routine strains of *Staph epidermidis* were examined for the presence of the group polysaccharide antigens A, B, and AC. In this material the majority of the presumed saprophytic nose strains belonged to group B whereas group AC was the most common among presumably pathogenic urinary strains.

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The significance of the line designated I (Fig. 3) is at the moment rather obscure, but the antigen involved seems to be widely distributed among the strains examined. The question arises whether this antigen may play a part in some of the cross reactions observed between strains from different groups.

Due to the chemical composition (7) and antigenic properties of their polysaccharides, the strains 1254 and 1268 are regarded as group II strains, i.e. type B strains of Julianelle & Wiegand (6). It may be reasonable to use the designation AC for the serologic reactivities demonstrated in polysaccharides 1622 and 3519, until the chemical nature of the active groups has been definitely determined.

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## TUBERCULIN PRODUCTION

### 2 Comparison between Amount of Tuberculin Obtained from the Culture Filtrate and by Extraction of Tubercle Bacilli

By

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Studies on extraction of tuberculoprotein and tuberculin from *Mycobacterium tuberculosis* have been reported frequently (Koch 1897, Coghill 1926 a, 1926 b, Gaugh 1933, Heidelberger & Menzel 1934, Menzel & Heidelberger 1938, Arena 1938, Gronwall 1944, Heckly & Watson 1950, 1951, Nagao & Nakajima 1951, Seibert & Fabrizio 1952, Baldwin et al 1953, Ito 1953, Ito et al 1953, Ito & Imaki 1954, Imaki 1955, Ohtomo 1955, Seiberl, Figueroa & Dufour 1955, Billaudelle & Warfvinge 1957, Paraf et al 1959, Shindo & Murai 1960, Tirunarayanan et al 1960, 1961, Shindo 1961, Ide et al 1961, Yugi et al 1962). Usually living virulent bacteria have been extracted, and the extraction performed with sodium hydroxide, phosphate or borate buffer, saline, or with water in combination with freezing and thawing (Tirunarayanan et al 1960, 1961). Urea has also been used (Seibert & Fabrizio 1952, Baldwin et al 1953). The yield and the biological activity of the extracts in skin tests vary considerably in these previous studies, and only few of them include a comparison with the amount of tuberculin in the culture filtrates. The main purpose of the present study is direct comparison under varying experimental conditions, of the amounts of tuberculoprotein and tuberculin in the culture filtrates of *M. tuberculosis* and the amounts which can be extracted from the cells.

In preliminary (unpublished) experiments with heat-killed cultures of *Mycobacterium tuberculosis*, larger amounts of tuberculoprotein were extracted with weak sodium hydroxide than with saline or phosphate buffer. Extraction was slow at room temperature but fairly quick at higher temperatures. It was also found that the biological activity of tuberculin could be destroyed by prolonged treatment with sodium hydroxide. Therefore, in the present study extraction was carried out with weak sodium hydroxide (0.05 N).

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## TUBERCULIN PRODUCTION

### 2 Comparison between Amount of Tuberculin Obtained from the Culture Filtrate and by Extraction of Tubercle Bacilli

By

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M. WEIS BENTZEN

Received 15/1/63

Studies on extraction of tuberculoprotein and tuberculin from *Mycobacterium tuberculosis* have been reported frequently (Koch 1897, Coghill 1926 a, 1926 b, Gaugh 1933, Heidelberg & Menzel 1934, Menzel & Heidelberg 1938, Arena 1938, Gronwall 1944, Heckly & Watson 1950, 1951, Nagao & Nakajima 1951, Seibert & Fabrizio 1952, Baldwin *et al* 1953, Ito 1953, Ito *et al* 1953, Ito & Imaki 1954, Imaki 1955, Ohtomo 1955, Seibert, Figueroa & Dufour 1955, Billaudelle & Warfvinge 1957, Paraf *et al* 1959, Shindo & Murai 1960, Tirunarayanan *et al* 1960, 1961, Shindo 1961, Ide *et al* 1961, Yugi *et al* 1962). Usually living virulent bacteria have been extracted, and the extraction performed with sodium hydroxide, phosphate or borate buffer, saline, or with water in combination with freezing and thawing (Tirunarayanan *et al* 1960, 1961). Urea has also been used (Seibert & Fabrizio 1952, Baldwin *et al* 1953). The yield and the biological activity of the extracts in skin tests vary considerably in these previous studies, and only few of them include a comparison with the amount of tuberculin in the culture filtrates. The main purpose of the present study is direct comparison under varying experimental conditions, of the amounts of tuberculoprotein and tuberculin in the culture filtrates of *M. tuberculosis* and the amounts which can be extracted from the cells.

In preliminary (unpublished) experiments with heat-killed cultures of *Mycobacterium tuberculosis*, larger amounts of tuberculoprotein were extracted with weak sodium hydroxide than with saline or phosphate buffer. Extraction was slow at room temperature but fairly quick at higher temperatures. It was also found that the biological activity of tuberculin could be destroyed in prolonged treatment with sodium hydroxide. Therefore, in the present study extraction was carried out with weak sodium hydroxide (0.05 N).

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In preliminary (unpublished) experiments with heat killed cultures of *Mycobacterium tuberculosis* larger amounts of tuberculoprotein were extracted with weak sodium hydroxide than with saline or phosphate buffer. Extraction was slow at room temperature but fairly quick at high or low

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The four factors referred to in this plan are Strain age of culture and fraction, all at two levels and medium at three levels.

The assay included 96 guinea pigs and each animal received eight tuberculin injections six test tuberculins selected according to the plan and the two standard dilutions. Thus every test tuberculin was used for 24 injections.

**Tuberculin injection** The injections were given into eight sites located in a zigzag row on the two flanks. All injections were given intradermally by the same person and the volume injected was 0.2 ml.

**Reading** The reactions were read after 24 and 48 hours. The size of the erythema (in mm) was measured with a ruler and the average of two diameters at right



formula

$$\log D_{obs} = \frac{TR_T - TR_{ST}}{b_{ST}} + 1.5$$

where  $\overline{TR_T}$  = average tuberculin reaction for the test tuberculin

$\overline{TR_{ST}}$  = average tuberculin reaction for the two standard doses and

$b_{ST}$  = average slope of the tuberculin dose-response curve = difference

between the three groups. For each of the three groups

estimates of the standard error (SE) of  $\log D_{obs}$  are obtained from these sets of three values.

The activity  $D$  (in units per ml) of the culture filtrates is calculated from  $D = 1000 \times \overline{D_{obs}}$  and the activity of the extracts from  $D = 1000 \times \overline{D_{obs}} \times \frac{1}{10}$  (dilution factor) where  $\overline{D_{obs}}$  is the geometric mean of the three determinations of  $D_{obs}$ .

The 95 per cent confidence limits for the activity are  $D \times 10^{\pm 2.58}$ .

## RESULTS

**Protein analyses** Table 1 shows the dry weight of organism (in mg per flask) for the cultures and the protein yields (in mg) for the culture filtrates and the extracts, calculated per ml of culture filtrate.

Taking into consideration that the protein analyses for the glycerol broth culture filtrates are not comparable with the other protein analyses since they include the meat protein of the broth, (Magnusson, Kim & Bentzon 1963), it will be seen from the table that more protein was extracted from the cells than was present in the culture filtrates, except for strain F-9606 cultured on Lind hill medium.

More protein was extracted from 8 weeks-old cultures than from 4 weeks-old cultures.

## Strain age and Lind hill medium

The average slope was used because there were only random variations in the slopes for the individual animals.

## MATERIALS AND METHODS

**Cultures** Two virulent strains of *Mycobacterium tuberculosis* T3487 and F9656 were cultured at 38° C for four and eight weeks in small flasks containing 180 ml of glycerol broth Sauton or Lind bli medium (Lind 1948). The cultures were sterilized by heating in streaming steam for one hour. The bacteria were filtered off on paper and dried before extraction. The cultures were included in a previously published study (Magnusson Kim & Bent on 1963) where further details are given regarding the cultures.

**Extraction** 200 mg of dried cells were suspended in 7 ml of 0.05 N sodium hydroxide and boiled for 30 minutes, cooled and centrifuged. 5 ml of the supernatant was drawn off by pipette while another 5 ml of 0.05 N sodium hydroxide was added to the bacterial suspension and the extraction repeated. To prevent destruction of the tuberculin activity, part of each extract was immediately diluted with buffer and sterilized by autoclaving before being used in the biological assay. Another part of the extract was precipitated with trichloroacetic acid for protein determination.

In all five extractions of each bacterial sample were carried out. After all the extractions were finished, the extracts originating from the same bacterial specimen were mixed and used for the biological assay or protein determination.

**Protein determination** Determination of protein was made by the biuret reaction as described previously (Magnusson Kim & Bent on 1963). For the culture filtrates

the protein yield is calculated as follows: Protein yield (in mg per ml) =  $\frac{F}{0.110} \times \frac{1}{10}$  ( $F$  = extinction observed, 0.110 = extinction for 1 mg/ml protein and  $\frac{1}{10}$  is a dilution factor).

For the extracts, the protein yield (per ml of culture filtrate) is calculated from protein yield (in mg per ml) =  $\frac{F}{0.110} \times 5 \times \frac{W}{200} \times \frac{1}{150}$  (5 is the dilution factor for

extracts and  $\frac{1}{150} \times \frac{W}{200}$  is due to the extraction being made on 200 mg of a total of  $W$  mg cells obtained from 150 ml culture filtrate, 150 ml is an estimate of the average amount of culture filtrate obtained from 180 ml medium).

**Biological assay** The biological assay included a comparison of the activity of the 12 extracts and the corresponding culture filtrates by intradermal testing on BCG vaccinated guinea pigs. These 24 preparations were compared with tuberculin dilutions containing 10 tuberculin units (TU) and 100 TU per 0.2 ml. Purified tuberculin RT 22 from Statens Seruminstitut was used as standard tuberculin. 1 mg of RT 22 was assumed to be equal to 75 000 TU.

**Tuberculin dilutions** The intention was to use such concentrations of the test tuberculin as would give skin reactions of a size between the reactions to the two standard doses. Preliminary assays revealed that appropriate reactions could be obtained when the culture filtrates were diluted 1:200 and the extracts 1:24000.

Fresh dilutions were prepared on each testing day. Sterile phosphate buffered saline was used as diluent. (One litre sterile buffer contains 145 g  $\text{KH}_2\text{PO}_4$  + 760 g  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  + 48 g  $\text{NaCl}$ ).

**Experimental animals** White BCG vaccinated guinea pigs weighing 530-650 g were used. Each animal was vaccinated intracutaneously in four places on the abdomen with 0.1 ml of Danish BCG vaccine, ten times stronger than the standard vaccine. Thus each animal received 3 mg of (semi dried weight) BCG. The vaccine batches were Nos 1141-1143. The animals were used five to six weeks after vaccination.

**Experimental design** It is known that the tuberculin sensitivity in a group of sensitized animals varies from one animal to another and even within an animal the sensitivity varies according to the test site. Therefore in order to ensure accurate comparison between all the 24 test preparations with a limited number of animals, a special experimental design was used. The design (not shown) is a modification of that described in Cochran & Cox (1955) *Experimental Designs* p. 216 Plan 6.10.

1 The authors are grateful to Dr A. Tønderlund and Miss K. Bunch Christensen, BCG Department, Statens Seruminstitut for preparing and supplying the vaccine.

TABLE 2

Mean Size of Intradermal Reactions Read after 24 Hours (Erythema in mm) of BCG Vaccinated Guinea Pigs and Tuberculin Yields in Tuberculin Units per ml of Culture Filtrate and Extracts of two Strains of *Mycobacterium tuberculosis* Cultured on three Different Media

Strain	Culture		Culture filtrate		Extract	
	Medium	Age of culture (weeks)	Mean reaction* 24 hours (mm)	Tuberculin yield † (TU)	Mean reaction ‡ 24 hours (mm)	Tuberculin yield † (TU)
T3487	Glycerol broth	4	16.6	25 000	15.1	1 300
		8	19.4	80 000	18.1	4 500
	Sauton	4	18.8	60 000	18.2	4 500
		8	19.4	80 000	20.3	11 000
	Lind hill	4	18.1	45 000	17.4	3 500
		8	19.8	90 000	20.2	11 000
E9636	Glycerol broth	4	20.0	100 000	17.2	3 000
		8	20.2	110 000	18.1	4 500
	Sauton	4	17.7	40 000	21.1	10 500
		8	18.9	67 000	20.5	12 500
	Lind hill	4	20.1	100 000	19.2	7 000
		8	21.6	190 000	20.7	13 500

Mean reaction (96 reactions) to standard solution containing 100 TU / 0.2 ml 20.0 mm  
 Mean reaction (96 reactions) to standard solution containing 10 TU / 0.2 ml 14.5 mm

\* Each figure is the mean of 24 reactions. Dilution rate 1:200

† Each figure is the mean of 24 reactions. Dilution rate 1:24000 see text

‡ The 95 per cent limits of D are  $0.70 \times D$  and  $1.43 \times D$

\* Calculated per ml of culture filtrate

graphically in Fig. 1. As regards the extracts (Fig. 1, upper part), the points are located on a line with slope 1. This means that the biological activity is proportional to the amount of extracted protein, or in other words that all the extracts show the same biological activity per mg protein.

However, as regards the culture filtrates (Fig. 1, lower part) the points do not fit a line with slope 1. Generally, when much protein is formed this is less active (per mg) than when the amounts are smaller.

*Relationship between total amount of tuberculin and growth of the bacteria.* In order to test the relationship between the total amount of tuberculin obtained from a culture and the weight of the culture, the logarithm of the total tuberculin activity ( $T_{et} + T_{ex}$ ) for each culture has been calculated. In Fig. 2 these values have been plotted against the weight of the cultures.

Even though there is some relationship between the total amount of tuberculin formed and the weight of the culture, the deviations of the points from a straight line are larger than might be expected with the actual experimental error.

TABLE 1

*Dry Weight of Organism (in mg) and Protein Yields (in mg per ml) in Culture Filtrates and Extracts of two Strains of Mycobacterium tuberculosis (cultured on three Different Media)*

Strain	Culture		Dry weight of organism per flask* (mg)	Protein yield (mg)	
	Medium	Age of culture (weeks)		Culture filtrate	Extract†
T1487	Glycerol broth	4	330	0.33§	0.13
		8	820	0.50§	0.43
	Sauton	4	1350	0.16	0.41
		8	1350	0.16	1.25
	Land hill	4	1000	0.16	0.35
		8	2100	0.36	1.34
F9656	Glycerol broth	4	570	0.19§	0.31
		8	820	0.09§	0.62
	Sauton	4	1500	0.07	0.67
		8	1320	0.19	0.92
	Land hill	4	1900	0.73	0.60
		8	2000	1.43	1.45

\* 180 ml medium

§ Unreliable, includes meat protein from glycerol broth

† Calculated per ml of culture filtrate, see text

**Biological activity** The amount of tuberculin obtained by extraction of the cells is smaller than that present in the culture filtrate (Table 2). On an average, extraction yields only about 10 per cent of the amount of tuberculin present in the culture filtrates. The percentage depends on the age of culture, strain and kind of medium, varying from about 3 per cent (4 weeks' culture of F9656 on glycerol broth) up to 27 per cent (4 weeks' culture of T9656 on Sauton medium).

More tuberculin was obtained from 8 weeks-old than from 4-weeks-old cultures. Land hill medium gave systematically higher yields of tuberculin than glycerol broth, and there were no systematic differences in the yield between the two strains.

The yields calculated on the basis of observations after 48 hours (Table 3) were systematically lower than after 24 hours (Table 2). However, the relationship between the test tuberculins was much the same after 48 hours as after 24 hours. The use of one set of average values ( $\bar{b}_{ST}$  and  $\bar{t}_{RST}$ ) for all the 24 hour comparisons and another set for all the 48-hour comparisons may account for the systematic trend. This difference has not been observed in later studies.

**Relationship between protein content and biological activity** The logarithms of the amount (in mg) of protein per g of dried weight of organism or per ml culture filtrate, and the tuberculin activity (in TU) per g of dried weight of organism or per ml culture filtrate, have been calculated for the 24 tuberculin preparations. The results are shown



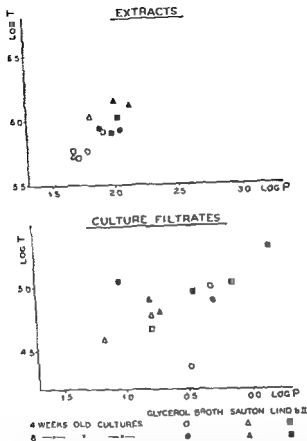


Fig 1

Tuberculin activity in relation to protein content of extracts and culture filtrates of *Mycobacterium tuberculosis* cultured on three different media

Abscissa Logarithm of the protein content in mg per g of dried weight of organism or per ml culture filtrate

Ordinate Logarithm of the tuberculin activity in TU per g of dried weight of organism or per ml culture filtrate

was much bigger from Lind bII than from Sauton medium. However, the biological activity of the preparation per mg substance was correspondingly lower, and there was little difference in the total tuberculin activity obtained on the two media. In the present experiment, a higher yield of tuberculin was obtained on Lind bII medium than on glycerol broth. Usually the tuberculin yield was also higher on Lind bII medium than on Sauton. The average yield on Sauton was about 60 per cent of that on Lind bII. The yields of tuberculin differ less than the yields of tuberculoprotein on the two media.

According to Green (1946), the protein content is a reliable measure of the biological activity of tuberculin preparations. However, Seibert,

TABLE 3

Mean Size of Intradermal Reactions Read after 48 Hours (Erythema in mm) of BCG Vaccinated Guinea Pigs and Tuberculin Yields (in Tuberculin Units per ml) of Culture Filtrates and Extracts of two Strains of *Mycobacterium tuberculosis* Cultured on three Different Media

Strain	Culture		Culture filtrate		Extract	
	Medium	Age of culture (weeks)	Mean reaction* 48 hours (mm)	Tuberculin yield† D (TU)	Mean reaction‡ 48 hours (mm)	Tuberculin yield† D (TU)
T3487	Glycerol broth	4	13.1	15 000	11.0	800
		8	16.7	60 000	15.4	7 500
	Sauton	4	16.2	50 000	15.5	4 000
		8	16.4	50 000	17.6	9 000
	Land III	4	15.5	40 000	14.4	2 500
		8	17.6	80 000	18.0	9 000
T9656	Glycerol broth	4	17.1	65 000	14.9	3 000
		8	17.1	65 000	16.3	5 000
	Sauton	4	15.2	35 000	17.6	8 000
		8	16.3	50 000	17.6	8 000
	Land III	4	17.3	70 000	16.3	5 000
		8	19.3	140 000	18.2	9 500

Mean reaction (96 reactions) to standard solution containing 100 TU/0.2 ml 17.3 mm

Mean reaction (96 reactions) to standard solution containing 10 TU/0.2 ml 10.7 mm

\* Each figure is the mean of 24 reactions. Dilution rate 1:200

‡ Each figure is the mean of 24 reactions. Dilution rate 1:  $\frac{24000}{W}$  — see text

† The 95 per cent limits of D are  $0.67 \times D$  and  $1.49 \times D$

\* Calculated per ml of culture filtrate

## DISCUSSION

Arena (1938) made direct comparison between the amount of tuberculin extracted from the cells and the amount present in the culture filtrate and found a larger amount in the extracts. In the present experiment, fairly large amounts of tuberculoproteins have been extracted from tubercle bacilli. The biological activity of the extracts, calculated per mg protein, is constant but so small that the total activity of the extracts is smaller than the activity of the corresponding culture filtrates. Consequently, it is of no advantage to prepare tuberculin by extraction of the cells by means of the present extraction method. A modified procedure for extraction is to alkalinize the medium after growth of the bacilli and extract the tuberculin from the cells by subsequent heat sterilization of the cultures. In this way, the culture filtrate will include the extracted proteins. This procedure has been used in a subsequent study in this series.

Land (1948) found that the yield (by weight) of purified tuberculin

and the tuberculin activity of the extracts were compared with the protein content and the activity of the corresponding culture filtrates.

Large amounts of protein were extracted from the cells, but the biological activity of the extracts was low. The average activity of the extracts was ten times smaller than the activity of the culture filtrates.

On the whole, more tuberculin was obtained on Lind hill medium than on glycerol broth and Sauton medium. Under varying experimental conditions, the amount of tuberculin obtained from 8-weeks-old cultures was larger than from 4-weeks-old cultures. The relationship of the amount of tuberculin obtained with the two strains was dependent on the medium used.

As regards the extracts, all the protein had the same biological activity, as regards the culture filtrates, the activity was higher in one part of the protein than in another.

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Fig. 2

Total tuberculin activity of culture filtrates and extracts in relation to bacterial crop of *Mycobacterium tuberculosis* cultured on three different media

Abscissa: Logarithm of the dried weight of organism (in mg) per flask

Ordinate: Logarithm of the total tuberculin activity ( $T_{cf} + T_{ex}$ ) in tuberculin units per ml of culture filtrate

Figuera & Dufour (1955) have isolated tuberculoproteins with different biological activity per mg of protein, partly from unheated culture filtrates, and partly by extraction of tubercle bacilli. In the present experiment, variations in the protein content of culture filtrates and extracts were obtained by using three different media, two strains and cultures of different ages. The results indicate that some relation exists between protein content and tuberculin activity. However, this is no simple relationship, since not all the protein has the same biological activity. For instance, as mentioned above, the protein of Lind bII medium is not as active as that of Sauton medium, and the average activity of the extracts is only 5–10 per cent of that of the culture filtrates when calculated on the basis of the same amount of protein. Thus, the present experiment shows that protein content cannot be used as a measure of the tuberculin activity under varying experimental conditions.

#### SUMMARY

Two strains of *Mycobacterium tuberculosis* were cultured for four and eight weeks on glycerol broth, Sauton and Lind bII medium. After heat sterilization of the cultures, the dried bacterial cells were extracted with 0.05 N sodium hydroxide. The amount of protein extracted

## POLYOMA VIRUS

### 1 Cell-Virus Interaction at Low Temperature

By

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The rate of adsorption of SE polyoma to primary mouse embryo cells at 37° C was determined by Winocour & Sachs (1) Using a plaque assay they found a maximum adsorption after 3 hours Crawford (2), applying the same technique on the Toronto strain of polyoma virus, found no difference in the amount of virus adsorbed at 4°, 22°, and 37° C

Deitchman (3) investigated the dynamics of SE polyoma virus multiplication in mouse embryo cells with cell virus suspensions which had been incubated for 18 hours at 4° C to ensure maximum virus adsorption No details are given to substantiate the validity of this procedure The present report deals with experiments intended to increase the yield of SE polyoma virus when grown in primary mouse embryo cells The effect of the temperature and of the incubation time for the initial cell virus interaction has been investigated in relation to the subsequent production of virus haemagglutinins by incubation of the infected cells at 37° C

### MATERIALS AND METHODS

**Virus.** A subline of SE polyoma virus was used for all experiments The virus has undergone several passages in monolayer cultures of primary mouse embryo cells The source of virus was infectious tissue culture fluid clarified by low speed centrifugation

**Cell cultures.** Primary mouse embryo cells were prepared by trypsinization of minced mouse embryo tissue The cells were grown in 250 ml medium or in 600 ml medium The cells were confluent in 1 day at 37° C Eagle flasks as changed to

When the cell virus interaction had taken place for a desired time the virus

Originally obtained from Professor Herbert Morgan University of Rochester, and designated SE polyoma virus LS 11

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TABLE 2

*Effect of Initial Cell-Virus Interaction Temperature on Early Productions of Haemagglutinins*

Interaction temperature	Extracellular HA on day				Intracellular HA on day	
	HA titre			Total HA units*	HA titre	Total HA units
	0	1	2		2	2
4°	05	0	64	4800	256	2560
					128	1280
22°	0	0	64	4800	64	640
			32	2400	32	320
37°	0	0	8	600	8	0
			0	0		

\* (HA units per ml)  $\times$  volume      ‡ Less than 8

Each prescription bottle was inoculated with 7.5 ml of a virus suspension with a HA titre 1/206. The inoculum was replaced with 15 ml medium for virus growth. Intracellular HA was estimated after freezing and thawing the cells in 2 ml PBS.

intracellular haemagglutinins was found after 10 days in the 4° and 22° C cultures. The yield after incubation at 37° C is lower than the corresponding yields obtained after incubation at 4° and 22° C. This is the case for both extracellular haemagglutinins after various incubation times and for the intracellular haemagglutinins determined after 10 days.

A second experiment was made to investigate whether the early detectable difference in the amount of extracellular haemagglutinins in the 4°, 22°, and 37° C cultures was due to a different rate of release of virus from the cells. Table 2 shows the results of haemagglutinin titrations at the time of the first appearance of extracellular haemagglutinins. There is a difference in the amount of both extracellular and intracellular virus, with a maximum yield in the cultures where interaction took place at 4° C.

#### *Effect of Cell-Virus Interaction Time on Virus Yield*

The relationship between time and virus yield was investigated. The experiments were made with cells grown in prescription bottles, and the interaction time was varied from 1 to 300 minutes at 4° C and from 1 to 180 minutes at 22° C. Table 3 shows the results when the cell-virus interaction took place at 4° C. Higher titres both of extracellular and intracellular haemagglutinins are found when the time is increased from 1 to 20 minutes. Prolonged interaction time had no significant effect on the virus yield. The appearance of extracellular haemagglutinins at zero time in cultures with an initial interaction time of 180 and 300 minutes indicates, however, that the amount of virus attached to the cells may increase beyond 20 minutes.

Table 4 shows the results from a similar experiment carried out at 22° C. A time interval from 40 to 180 minutes gave the same amount of

suspension was removed and the cell layer and the adjoining glass surfaces were carefully washed two times with Eagle Hanks' medium with 2 per cent horse serum. The volume of each washing was 7.5 ml and 25 ml respectively for the prescription bottles and the Roux bottles.

Eagle Hanks' medium with 2 per cent horse serum was then added and the cultures were incubated at 37° C.

*Estimation of virus.* Extracellular and intracellular haemagglutinins were estimated. The extracellular haemagglutinins were determined in the medium after heating at 56° C for 30 minutes.

To release intracellular haemagglutinins the monolayer was trypsinized, the cells spun down at low speed centrifugation and the sediment suspended in phosphate buffered saline of pH 7.2 (PBS). The suspension was frozen and thawed four times and then clarified by low speed centrifugation. The supernatant was heated at 56° C for 30 minutes prior to titration.

Haemagglutinations were made by adding 0.2 ml of a 0.4 per cent suspension of guinea pig erythrocytes to 0.2 ml of serial two fold dilutions of a virus suspension. PBS was used as diluent. The erythrocytes were suspended in PBS containing 3 per cent of a 30 per cent solution of bovine serum albumin. The haemagglutination was read after incubation at 4° C overnight.

## RESULTS

### *Effect of Cell-Virus Interaction Temperature on Virus Yield*

The effect of the initial cell-virus interaction temperature was studied by infecting primary cell cultures with virus suspensions at 4°, 22°, and 37° C. After 3 hours interaction at the respective temperatures the virus suspension was substituted with medium and the incubation continued at 37° C. Aliquots of the medium for titrations of the zero time extracellular haemagglutinins were taken after 30 minutes to allow any elution to occur before sampling. Table 1 shows the effect of temperature variations on the virus production. The yield of extracellular virus in cultures where the interaction took place at 4° C is identical to that obtained at 22° C when compared after incubation for 2, 8, and 10 days. Slightly higher titres were observed after 4, 5, and 6 days in the 4° C cultures. No significant difference in the amount of

TABLE 1  
*Effect of Initial Cell-Virus Interaction Temperature on Production of Haemagglutinins (HA)*

Inter- action tempe- rature	1 virg cellular HA on day							Intracellular HA on day		
	HA titre							Total HA units*	HA titre	Total HA units
	0	2	4	5	6	8	10	10	10	10
4°	2	64	128	128	256 128	256	256	64000	4096 2048	61440 30720
22°	0.5	64	64	64	128	256	256	64000	4096	61440
37°	0	16 32	32	16	64	128	128	32000	1024	15360

\* (HA units per ml) × volume

‡ Less than 2

Each Roux bottle was inoculated with 20 ml of a virus suspension with a HA titre 1/128. The inoculum was replaced with 50 ml medium for virus growth. Intracellular HA was estimated after freezing and thawing the cells in 3 ml PBS.



time used for the initial cell-virus interaction. When titrations were made on infected cultures which had been incubated for 1-10 days, a constantly lower titer was obtained when the interaction took place at 37° C compared with that obtained at 4° and 22° C. The effect was not due to a decreased release of haemagglutinins as lower titres were obtained by estimation of both extracellular and intracellular haemagglutinins. A difference in haemagglutinin titres was less pronounced by comparison of cultures kept at 4° and 22° C. Significant higher titres were however found as an early event in the cultures where the initial cell virus interaction took place at 4° C. This was especially the case for the intracellular haemagglutinins. The experiments therefore indicate that a better yield of early virus is obtained when the temperature for the initial interaction of the virus with the cell is lowered from 37° to 4° C.

The total amount of virus attached to cells in these experiments was too small to be detected by haemagglutinin titrations of the inoculum before and after attachment had taken place. The experiment presented in Table 1 indicated, however, that some release of haemagglutinins occurred at 37° C from cells which had been kept at 4° C while no such release could be demonstrated with cells kept at 22° or at 37° C. The release could be explained by two assumptions:

- a) a greater amount of virus being adsorbed at the lower temperature followed by
- b) release of part of the adsorbed virus by elevating the temperature due to thermolability of the cell-virus binding.

The increased virus yield by interaction at 4° C would then indicate that attachment with subsequent infection of the cells prevails above release compared with the effects of interaction which takes place at higher temperatures. A more optimal infection of the cells could possibly be achieved by further studies on the effect of virus yield of variations in the rate of temperature change from 4° to 37° C.

At low temperatures the Brownian movements of suspended virus particles are decreased due to the higher viscosity of the suspending medium (4). The number of virus particles colliding with the cells per unit time is therefore reduced. An increased stability of the cell-virus bond obviously counteracts and exceeds the effect of viscosity change and is therefore probably responsible for the increased virus yield when the cell-virus interaction takes place at low temperature.

As shown by Table 3 and 4, an attachment time of only 20 minutes at 4° C or 40 minutes at 22° C, gave maximal yield of virus haemagglutinins. The results indicate, however, that there may be a time dependent attachment beyond 20 and 40 minutes. The experiments by Winocour & Sachs (1) showed that an attachment time of 3 hours at 37° C was required to obtain a maximum number of plaques.

haemagglutinins. A slightly decreased yield was obtained after interaction for only 1 to 20 minutes. The extracellular haemagglutinins found on the second day in cultures with an initial interaction time of 60 and 180 minutes may indicate that such prolonged interaction has some effect on the virus production.

TABLE 3

*Effect of Initial Cell-Virus Interaction Time at 4° C on Early Production of Haemagglutinins*

Interaction time (min.)	Extracellular HA on day				Intracellular HA on day	
	HA titre			Total HA units* §	HA titre	Total HA units
	0	1	2		2	2
1	0§	0	64	4800	64	640
20	0	0	256	19200	256	2560
40	0	0	256	19200	512	5120
					256	2560
60	0	0	256	19200	512	5120
					256	2560
180	8	0	256	19200	512	5120
300	8	0	512	38400	512	5120
			256	19200		

\* (HA units per ml) × volume

§ Less than 8

Each prescription bottle was inoculated with 7.5 ml of a virus suspension with a HA titre 1/256. The inoculum was replaced with 15 ml medium for virus growth. Intracellular HA was estimated after freezing and thawing the cells in 2 ml PBS.

TABLE 4

*Effect of Initial Cell-Virus Interaction Time at 22° C on Early Production of Haemagglutinins*

Interaction time (min.)	Extracellular HA on day					Intracellular HA on day	
	HA titre				Total HA units* §	HA titre	Total HA units
	0	1	2	3		3	3
1	0§	0	0	32	2400	16	160
				16	1200		
20	0	0	0	64	4800	32	320
				32	2400	16	160
40	0	0	0	64	4800	64	640
60	0	0	8	64	4900	64	640
						32	320
180	0	0	8	64	4800	128	1280
						64	640

\* (HA units per ml) × volume

§ Less than 8

Each prescription bottle was inoculated with 7.5 ml of a virus suspension with a HA titre 1/256. The inoculum was replaced with 15 ml medium for virus growth. Intracellular HA was estimated after freezing and thawing the cells in 2 ml PBS.

## DISCUSSION

The investigations show that the appearance of haemagglutinins produced at 37° C by monolayers of primary mouse embryo cells infected with SE polyoma virus depends upon the temperature and the

time used for the initial cell-virus interaction. When titrations were made on infected cultures which had been incubated for 1-10 days, a constantly lower titer was obtained when the interaction took place at 37° C compared with that obtained at 4° and 22° C. The effect was not due to a decreased release of haemagglutinins as lower titres were obtained by estimation of both extracellular and intracellular haemagglutinins. A difference in haemagglutinin titres was less pronounced by comparison of cultures kept at 4° and 22° C. Significant higher titres were however found as an early event in the cultures where the initial cell virus interaction took place at 4° C. This was especially the case for the intracellular haemagglutinins. The experiments therefore indicate that a better yield of early virus is obtained when the temperature for the initial interaction of the virus with the cell is lowered from 37° to 4° C.

The total amount of virus attached to cells in these experiments was too small to be detected by hamagglutinin titrations of the inoculum before and after attachment had taken place. The experiment presented in Table 1 indicated, however, that some release of haemagglutinins occurred at 37° C from cells which had been kept at 4° C while no such release could be demonstrated with cells kept at 22° or at 37° C. The release could be explained by two assumptions:

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- b) release of part of the adsorbed virus by elevating the temperature due to thermolability of the cell-virus binding

The increased virus yield by interaction at 4° C would then indicate that attachment with subsequent infection of the cells prevails above release compared with the effects of interaction which takes place at higher temperatures. A more optimal infection of the cells could possibly be achieved by further studies on the effect of virus yield of variations in the rate of temperature change from 4° to 37° C.

At low temperatures the Brownian movements of suspended virus particles are decreased due to the higher viscosity of the suspending medium (4). The number of virus particles colliding with the cells per unit time is therefore reduced. An increased stability of the cell-virus bond obviously counteracts and exceeds the effect of viscosity change and is therefore probably responsible for the increased virus yield when the cell virus interaction takes place at low temperature.

As shown by Table 3 and 4, an attachment time of only 20 minutes at 4° C, or 40 minutes at 22° C, gave maximal yield of virus haemagglutinins. The results indicate, however, that there may be a time dependent attachment beyond 20 and 40 minutes. The experiments by Winocour & Sachs (1) showed that an attachment time of 3 hours at 37° C was required to obtain a maximum number of plaques.

*Martin & Work* (5) obtained a steeper growth curve of LMC virus in mouse ascites cells by rapid elevation of the temperature of the cell virus complex from 4° to 37° C. This effect was explained as being due to a thermal shock resulting in a fair degree of synchronous virus growth. A similar mechanism may be partly responsible for some of the increased virus yield recorded in our experiments. A thermal shock can, however, hardly account for the increased yield by attachment at 22° C compared with that obtained when the attachment took place at 37° C.

*Crawford* (2) found no increase in the plaque titres after adsorption at low temperatures. Further experiments are being performed to test if the advantage of the low temperature interaction reported in our experiments concerning haemagglutinin production can also be applied to plaque titrations.

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# DOUBLE DIFFUSION PRECIPITATION AND IMMUNOELECTROPHORESIS OF E. COLI B ANTIGENS

By

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Antigens of several bacterial strains have been studied with the aid of double diffusion precipitation (DDP) and immunoelectrophoresis (IE). A thorough investigation of mycobacterial antigens has been conducted by Lind (1961). He demonstrated by means of the DDP technique the presence of 17 different precipitinogens in a mycobacterial strain and investigated their occurrence in others. Certain physical chemical and immunological characteristics of these antigens were also reported. Hanson & Holm (1961) found 12 distinct antigens in the IE of streptococcal antigens identifying among them Dick's toxin, streptokinase and streptolysin. Felton *et al.* (1958) found a large number of antigens in the IE of ultrasonically disrupted preparations of *H. pertussis*. They suggested that some of their antigens might be products of enzymatic degradation of the native antigens. Furthermore Grabar (1960) found 9 precipitinogens in *Salmonella* while investigating O- and V<sub>1</sub> antigens.

It is evident that DDP and IE are excellent tools in the study of microbial antigens. We have applied these techniques to the analysis of antigens of *Escherichia coli*, which have been intensively investigated in the past with the aid of other immunological techniques. This paper describes some of the characteristics of the antigens of *E. coli* B as revealed by these techniques.

## METHODS

Antigen 4  
was grown  
in glucose  
shaking  
of the  
for 60 h  
pended  
2 x 1  
h  
f  
n

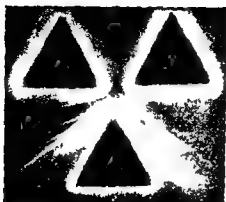


Fig 1

Precipitation patterns arising from the reaction between anti-*E* coli B serum and *E* coli B antigen material in ODP. Bacterial antigen is in the two upper reservoirs and the antiserum in the lower reservoir

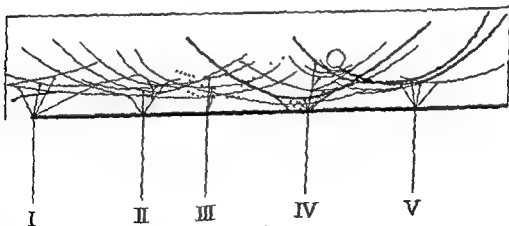


Fig 2

Immunoelectrophoresis of *E* coli B antigens. For explanations see text

For experiments with DDP and IF the disrupted cells were centrifuged at 18000 X g for 60 minutes. The supernatant which contained about 5 per cent of protein was used for precipitation tests.

**Immunization.** Four rabbits were immunized with the antigen preparation. Each rabbit was inoculated subcutaneously with an initial dose of 5 ml of antigen preparation which had been diluted 1:1 with complete Freund's adjuvant. The immunization was continued with 2 ml of antigen once a week intramuscularly. The increase in antibody titer was checked monthly. After 3 months the animals were sacrificed and the blood collected by cardiac puncture.

**Immunological tests.** Both macro and microtechniques of the DDP were used (Ouchterlony 1958). When the macro method was employed the basins were refilled 3-4 times before the incubation was started at room temperature.

The method for micro IE was that of Schetligger (1955). It was found convenient to carry out comparative IF (Walsworth & Hanson 1960) on the same plate as the conventional IE.

The precipitation patterns were recorded photographically after which the plates were dried and stained with Lissamine green.

## RESULTS

The DDP and IE patterns of *E. coli* B antigens with rabbit anti-*E. coli* B sera are seen in Figs. 1 and 2. In DDP about 12-13 bands are seen. The majority of the precipitation bands were formed close to the antibody reservoir. In IE up to 24 bands could be demonstrated. We have arranged the bands in five main groups as shown in Fig. 2. The first group consists of 12 bands. The second group consists of 1 band. The third group consists of 1 band. The fourth group migrates only slightly towards the anode and is composed of 6 bands. The fifth group, which consists of 4 bands, moved slightly towards the cathode.

## DISCUSSION

The good resolution of a large number of *E. coli* B antigens which was obtained with IE seems to invite other applications of the procedure. For instance, it would be interesting to compare the patterns of precipitation bands from several strains of *E. coli*, e.g. by utilizing the above mapping principle. Another logical extension of this investigation would be to try to correlate the antigens found in our studies with those revealed by other techniques.

In the present study no attempts were made to further fractionate the antigenic material. Therefore, a more detailed analysis of the antigens described in this paper could be pursued along the lines described by Bussard (1955).

## SUMMARY

*E. coli* B antigens have been studied with the aid of double diffusion precipitation and immunoelectrophoresis. The former technique reveals

12-13 antigens whereas 24 antigens can be observed by the latter procedure. The antigens found in IC have been divided into five groups.

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## AUTOCLAVING OF SYRINGES IN PRESSURE COOKERS<sup>1</sup>

By

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Syringes may be sterilized by dry heat or by moist heat. The former procedure is most often recommended for hospital use (1, 2). Among the advantages of the method are that the syringes can be sterilized assembled and in sealed packages, and the question of subsequent drying does not arise. Its disadvantages—particularly with regard to its use in general medical and nursing practice—are that the actual apparatus is relatively expensive, and the method can fail, because it is often difficult to judge when the temperature in the load has reached the required value. In addition, anything less than scrupulous cleaning of syringe and needle involves the risk of retaining fragments of organic matter which may burn together with the internal surfaces, thus compromising the effectiveness of subsequent mechanical cleaning. Finally, dry sterilization is a relatively protracted process.

Bacterial spores are killed most rapidly and effectively by using saturated steam at a temperature of 120° C and upwards. In recognition of this fact, autoclaving by means of an ordinary domestic pressure cooker has in the course of time been repeatedly advocated for general medical practice.

McCulloch (3) emphasizes the following advantages of this simple type of autoclave: an excess pressure of 1 atmosphere is reached very rapidly, corresponding to a saturated steam temperature of about 120° C; the relatively small volume of the container permits removal of the atmospheric air quite effectively by means of steam discharge; for a few minutes before closing the pressure valve, the sterilized material can be removed when still so hot that there is no drying problem. He concludes by saying: "While not to be considered as approximating the safety of sterilization in a properly designed steam pressure sterilizer, these cookers offer more positive protection than does boiling." Baumann (4) likewise recognizes the practicability of a simple pressure cooker and finds that its greatest disadvantage is that the load cannot

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<sup>1</sup> Part of this paper was published in *Lægeskr. Læg.* 125: 1131, 1962.

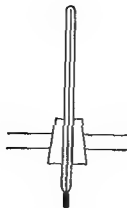


Fig 1

The lid of the pressure cooker with the thermometer. The excess pressure in the cooker squeezes the rubber stopper against the thermometer and the hole in the lid.

be removed in sterile packing, ready for use. *Jorgensen* (5) states that during autoclaving, syringes should be placed disassembled in open containers which are not closed until after sterilization, the effective duration of which should be 15-20 minutes.

The present authors have been unable to find any systematic study of the efficiency of the sterilizing process when syringes and needles are autoclaved in a household pressure cooker.

#### EXPERIMENTS

A domestic pressure cooker— with a volume of 6 litres was used in the experiments. To permit measurements of the temperature in the cooker's free space and in the actual load, a hole was bored in the lid to fit a rubber stopper carrying a mercury thermometer (Fig 1) and thermocouple leads for a thermoelectric meter. The load was placed on a rack in the cooker, so that it was not exposed to direct contact with the water in the bottom of the cooker.

Dried spores of *B. subtilis* (the strain used in spore preparations from Statens Serum Institut for routine control of autoclaves, dry sterilizers and formalin ovens) were used to determine the efficiency of the sterilizing process. The spore material was used in two forms: as test preparations, i.e. a mixture of spores with sterile sand, dried under vacuum and packed in small double-walled paper bags, or as a suspension of spores in broth to give a standard spore density. The latter was used to contaminate syringes and needles.

During the process of autoclaving, the temperature in the pressure cooker was checked by the thermoelectric meter mentioned, so that a continuous record could be made of the temperature, both in the free space of the cooker and in the load.

a) *Experiments with Spore Preparations and Different Obstructions to the Steam*

Test preparations containing  $1.5 \times 10^6$  dried spores of *B. subtilis* were placed in pairs in 11 test tubes (15 cm long, 2 cm diameter), which were closed in two different ways

- 1) with non absorbent cotton wool only ("cotton wool stopper")
- 2) with non absorbent cotton wool + gauze and a further cover consisting of one layer of tissue paper and one layer of brown wrapping paper ("cotton wool gauze-paper stopper")

The tubes were placed in wire baskets on the rack in the pressure cooker which contained 500 ml of water and autoclaved at  $120^\circ \text{C}$ . After autoclaving the spores were inoculated into broth, incubated at  $37^\circ \text{C}$  and examined daily for growth of *B. subtilis*.

TABLE 1

*Sterilization Experiments: Trials with Different Obstructions to the Steam*

Trial no	No of spore preparations per trial	Cotton wool stopper autoclaving for		Cotton wool-gauze paper stopper autoclaving for			
		5 min	10 min	5 min		10 min	
		growth after 30 days	growth after 30 days	growth after		growth after	
				30 days	60 days	30 days	60 days
1	2 x 6	0/12*	0/12*	0/12*	0/12*	0/12*	0/12*
2	2 x 6	0/12	0/12	0/12	0/12	0/12	0/12
3	2 x 6	0/12	0/12	3/12	5/12	0/12	0/12
4	2 x 6	0/12	0/12	7/12	8/12	0/12	0/12
5	2 x 6	0/12	0/12	1/12	1/12	0/12	0/12
6	2 x 6	4/12	0/12	5/12	7/12	0/12	0/12
7	2 x 6	2/12	0/12	4/12	5/12	0/12	2/12
8	2 x 6	3/12	0/12	2/12	2/12	0/12	0/12
9	2 x 6	2/12	0/12	2/12	2/12	0/12	0/12
10	2 x 6	0/12	0/12	2/12	2/12	0/12	0/12
Total		11/120	0/120	26/120	32/120	0/120	2/120

\* Numerator: Showing growth on reading

Denominator: Number of spore preparations examined

Table 1 shows the results of these experiments. No growth was observed after 30 days in the broth seeded with the test preparations from the tubes with simple cotton wool stopper and autoclaved for 10 minutes. When the period of autoclaving was only 5 minutes, growth was obtained from a total of 11 out of 120 spore preparations.

In trials with the greater obstruction to the steam, i.e. cotton wool-gauze paper stopper, autoclaving for 5 minutes resulted in growth in 26 out of the 120 spore preparations when the cultures were followed for 30 days. On extending the period of observation to 60 days, the number of preparations showing growth increased to 32 out of the 120

Following autoclaving for 10 minutes none of the spore preparations (0/120) gave rise to growth within 30 days, while growth appeared in two out of 120 when the observation period was extended to 60 days

#### b) *Experiments with Spore-Contaminated Syringes and Needles*

Syringes and needles were contaminated with approximately  $1 \times 10^6$  spores by drawing into the syringe unit (syringe + needle) an adequate amount of a suspension of *B subtilis* spores in broth which was kept at 2 to 5° C

The syringes were then disassembled, although the needle was left on the barrel, and the separate parts were left at room temperature for about 18 hours to allow the spore material to dry. In experimental series no IV, drying was done at the same temperature, but under vacuum

A total of four experimental series were carried out

I With unpacked syringes and needles, placed in an open Petri dish during autoclaving

II, III and IV In these series, syringe units were wrapped in two layers of paper, the inner a layer of coarse filter paper and the outer a layer of brown wrapping paper, fastened with a string. Prior to the packing, the needles were placed in tubes plugged with cotton wool

A total of 145 syringe units were autoclaved at 120° C, grouped into four series with 25, 100, 15 and 5, respectively. In series I and II the autoclaving time was 5 minutes, in series III and IV it was 10 minutes. To check that *B subtilis* was not present in the vegetative form but in form of spores, 30 syringe units were heated in the pressure cooker at 100° C (25 for 5 minutes, 5 for 10 minutes). Finally, a number of unheated control units were included in each experimental series to determine the degree of contamination (Experimental series nos. III and IV were run together, so that the 5 control units indicated in Table 2 under experimental series no. III were common to both series III and IV. Altogether a total of 40 control units was examined)

The number of surviving spores per syringe unit was determined by rinsing the syringe and needle with 10 ml of chilled broth. Ten-fold dilution series were prepared from the rinsings, and 0.1 ml portions of these were plated on blood agar for colony counting. Both blood agar plates and the remainder of the original rinsing fluid (broth) were incubated at 37° C. The broth was observed for growth for a period up to 30 days.

Table 2 shows the results of the experiments. In none of the trials was there any growth of *B subtilis* on plating from the rinsings from the syringe units which had been autoclaved at 120° C. Nor did the actual broth for rinsing show growth on observation for up to 30 days. The insignificant difference between the counts obtained on plating from the control syringe units and from the units which had been heated at 100° C indicated that the bacteria had been in the spore stage during the trials.

TABLE 2  
*Sterilization Experiments: Trials with Spore Contaminated Syringes and Needles*

Series	Wrapping of syringe	Heat treatment	Heating minutes	No. of syringes	No. of spores per unit*	Remarks
I	unwrapped	approx 120° C 100° C untreated control	5 5	5 × 5 1 × 5 3 × 5	0 2.5 × 10 <sup>6</sup> 3.8 × 10 <sup>6</sup>	The contaminated syringes dried overnight at room temperature
II	wrapped	approx 120° C 100° C untreated control	5 5	20 × 5 4 × 5 4 × 5	0 2.3 × 10 <sup>6</sup> 4.5 × 10 <sup>6</sup>	do
III	wrapped	approx 120° C 100° C untreated control	10 10	3 × 5 1 × 5 1 × 5	0 2.5 × 10 <sup>5</sup> 4.0 × 10 <sup>6</sup>	do
IV	wrapped	approx 120° C	10	1 × 5	0	The contaminated syringes dried overnight under vacuum

\* Mean count of spores per syringe unit (syringe + needle)

Following autoclaving for 10 minutes none of the spore preparations (0/120) gave rise to growth within 30 days, while growth appeared in two out of 120 when the observation period was extended to 60 days

#### b) Experiments with Spore-Contaminated Syringes and Needles

Syringes and needles were contaminated with approximately  $1 \times 10^8$  spores by drawing into the syringe unit (syringe + needle) an adequate amount of a suspension of *B subtilis* spores in broth which was kept at 2 to 5° C

The syringes were then disassembled, although the needle was left on the barrel, and the separate parts were left at room temperature for about 18 hours to allow the spore material to dry. In experimental series no IV, drying was done at the same temperature, but under vacuum

A total of four experimental series were carried out

I With unpacked syringes and needles, placed in an open Petri dish during autoclaving

II, III and IV In these series, syringe units were wrapped in two layers of paper, the inner a layer of coarse filter paper and the outer a layer of brown wrapping paper, fastened with a string. Prior to the packing, the needles were placed in tubes plugged with cotton wool

A total of 145 syringe units were autoclaved at 120° C, grouped into four series with 25, 100, 15 and 5, respectively. In series I and II the autoclaving time was 5 minutes, in series III and IV it was 10 minutes. To check that *B subtilis* was not present in the vegetative form but in form of spores, 30 syringe units were heated in the pressure cooker at 100° C (25 for 5 minutes, 5 for 10 minutes). Finally, a number of unheated control units were included in each experimental series to determine the degree of contamination (Experimental series nos III and IV were run together, so that the 5 control units indicated in Table 2 under experimental series no III were common to both series III and IV. Altogether a total of 40 control units was examined)

The number of surviving spores per syringe unit was determined by rinsing the syringe and needle with 10 ml of chilled broth. Ten fold dilution series were prepared from the rinsings, and 0.1 ml portions of these were plated on blood agar for colony counting. Both blood agar plates and the remainder of the original rinsing fluid (broth) were incubated at 37° C. The broth was observed for growth for a period up to 30 days

Table 2 shows the results of the experiments. In none of the trials was there any growth of *B subtilis* on plating from the rinsings from the syringe units which had been autoclaved at 120° C. Nor did the actual broth for rinsing show growth on observation for up to 30 days. The insignificant difference between the counts obtained on plating from the control syringe units and from the units which had been heated at 100° C indicated that the bacteria had been in the spore stage during the trials

TABLE II  
Sterilization Experiments Trials with Spore Contaminated Syringes and Needles

Series	Wrapping of syringe	Heat treatment	Heating minutes	No. of syringes	No. of spores per unit*	Remarks
I	unwrapped	approx 120° C 100° C untreated control	5 5	5 X 5 1 X 5 3 X 5	0 2.5 X 10 <sup>6</sup> 3.8 X 10 <sup>6</sup>	The contaminated syringes dried overnight at room temperature
II	wrapped	approx 120° C 100° C untreated control	5 5	20 X 5 4 X 5 4 X 5	0 2.1 X 10 <sup>6</sup> 4.5 X 10 <sup>6</sup>	do
III	wrapped	approx 120° C 100° C untreated control	10 10	3 X 5 1 X 5 1 X 5	0 2.5 X 10 <sup>6</sup> 4.0 X 10 <sup>6</sup>	do
IV	wrapped	approx 120° C	10	1 X 5	0	The contaminated syringes dried overnight under vacuum

\* Mean count of spores per syringe unit (syringe + needle)

## Temperature Measurements

During the experiments, the temperature in the free space of the pressure cooker was measured both by mercury thermometer and by thermocouple. The temperature within the load was measured by thermocouple.

In all cases, there was a satisfactory agreement between temperatures, that of the load reaching the same maximum value as that of the free space, the greatest delay in doing so being 1 minute.

## DISCUSSION

"Sterilization" of syringes and needles is in many cases performed by boiling. However, this method of disinfection can be considered justified only if the mechanical cleaning prior to boiling and the entire procedure for handling the syringes and needles are absolutely irreproachable.

As mentioned, autoclaving by pressure cooker has been proposed repeatedly as a solution to the problem of sterilization in general practice. The fact that the method has not found wider use is presumably due to an uncertainty as to its effectiveness, and also to an impression that it suffered from the disadvantage that the sterilized material could not be removed in a packing suitable for storage, and that subsequent drying was necessary. The classic form of packing in two layers of paper is well suited to autoclaving, and the experiments described here show that this packing can be used in a pressure cooker. Evacuation of the atmospheric air merely by blowing off steam before attaching the pressure valve is surprisingly effective.

Autoclaving of syringes + needles contaminated with approximately  $10^7$  spores resulted in complete destruction of the spores in five minutes. Assuming that the destruction of the bacteria is an exponential function of time, the above result implies an exceedingly great safety factor when a routine autoclaving period of 20 minutes is employed, since such an increase in the sterilization time will give theoretically a reduction factor of more than  $10^{20}$ .

The present experiments using test preparations of spores show that a small increase in the obstruction to the steam will result in a reduction in the efficiency of the sterilizing process.

The possibility cannot be excluded that barriers to the steam greater than those employed in the present experiments might result in failure of the method, even with boiling under pressure for 20 minutes. It is considered advisable, therefore, that the type of pressure cooker available should be controlled for its efficiency of sterilization, and that the method of packing to be used should likewise be checked. Control will be necessary

- 1) if the barriers to the steam are judged to exceed those of the present experiments,



- 2) if there is any doubt that the temperature in the cooker has reached  $120^{\circ}\text{C}$

Control can be maintained by placing 10 test preparations in the sites most inaccessible to steam and then boiling under pressure for 10 minutes. If all the preparations are found to be sterile a good safety margin can be reckoned with in routine sterilization for 20 minutes.

All pressure cookers used for sterilization should be fitted with a thermometer. The sterilization time should only be calculated from the moment the thermometer shows at least  $120^{\circ}\text{C}$  and sterilization should not be for less than 20 minutes. In the present experiments the observation made by McCulloch was also confirmed namely that the sterilized material can be removed at a stage when its heat content is so great that in most cases subsequent drying becomes unnecessary.

#### SUMMARY

An account is given of the efficiency of the sterilizing process in a household pressure cooker. With an autoclaving period of only 20 minutes and using wrapped syringe units (syringe + needle) contaminated with approximately  $10^6$  dried spores of *B. subtilis*, a reduction factor of about 10 was obtained.

The method appears suitable as a routine for sterilizing syringes in general medical and nursing practice.

It is concluded that a pressure cooker used for sterilization should always be fitted with a thermometer and a temperature of at least  $120^{\circ}\text{C}$  should be employed for 20 minutes.

The possibility of a control of the method is discussed.

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## BRIEF REPORT

### AFINITIES BETWEEN MORAXELLA SPP. AND A STRAIN OF NEISSERIA CATARRHALIS AS EXPRESSED BY TRANSFORMATION

By Kjell Bøvre

Catlin & Cunningham (1961) reported that *Neisseria catarrhalis* is distinct from other *neisseriae* in terms of DNA base composition and transformation. Marmur & Doty (1962) reported similar base composition of DNAs from *Moraxella bovis* and *Neisseria catarrhalis* (strain No 11 Catlin).

**Material and methods.** A strain of *Neisseria catarrhalis* (No 11 Catlin) was subjected to the action of crude DNA extracts from streptomycin resistant mutants of four strains of *Moraxella nonliquefaciens* and one strain of *Moraxella bovis*. All these *Moraxella* strains had previously been found representative for their respective species as expressed by isospecific and heterospecific transformation ratios in quantitative experiments including 21 *Moraxella nonliquefaciens* strains and four *Moraxella bovis* type strains (Bøvre). In addition one type strain of *Moraxella liquefaciens* was included in the experiments as donor of streptomycin resistance.

With slight modifications the procedure was that of Bøvre & Henriksen (1962). The controls included parallels with non marker DNA marker DNA treated with DNase before use and different extracts of the same donor strain. As a negative control also served DNA extracted from a streptomycin resistant mutant of *Neisseria meningitidis* which had proved active in intraspecific transformation.

**Results.** The strain of *Neisseria catarrhalis* was transformed to streptomycin resistance by DNAs from all the strains of *Moraxella nonliquefaciens* and *Moraxella bovis*. The transformation frequency was low but reproducible and distinct from the spontaneous mutation frequency in quantitative experiments with the ordinary 15 minutes DNA exposure. The ratios of intergeneric to homospecific transformation frequencies were of the order  $1:4 \cdot 10^5$  without significant difference between the effects of the two donor species (homospecific transformation frequencies ranged from one to two per cent of the recipient population). When DNase treatment after 15 minutes DNA exposure was omitted in additional non quantitative tests DNA was found to have an effect on the growing agar culture through the 6-7 hr phenotypic expression period giving rise to several hundreds of transformant colonies per plate as compared with 0-2 spontaneous mutant colonies per control plate.

The above by the recipient even when DNase treatment was omitted.

**Comment.** The above mentioned ratios of intergeneric to homospecific transformation frequencies in the reactions between *Moraxella* spp. and *Neisseria catarrhalis* may be compared with the ratios  $2:7 \cdot 10^3$  of heterospecific to homospecific transformation to streptomycin resistance between strains of *Moraxella nonliquefaciens* and *Moraxella bovis* (Bøvre). The importance of the low frequency intergeneric reactions reported is unknown. An extended study in this subject is therefore indicated.

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# INDEX

VOL 58 FASC 1-4 1963

Ahren C	Effect of diencephalic lesions on tests in adult rabbits	390
Astrom K E	Experiments on allergic encephalomyelitis	388
Åhlström C G	Rous sarcoma in mammals and ducks	153
Åhlström C G	Yvon Bergman and Bengt Fhrenberg Neoplasms in guinea pigs induced by an agent in Rous chicken sarcoma	177
Åhrén A and L Angervall	Action of ovarian hormones on the mammary glands of alloxan diabetic rats	161
Åhrén A and L Angervall	Mammary gland development in newborn offspring of alloxan diabetic rats	171
Ållas Yrjö Aito Lahti Jussi Saukkonen and Tapani Laitio	Double diffusion precipitation and immunoelectrophoresis of F coli B antigens	517
Angervall L and K Åhrén	Action of ovarian hormones on the mammary glands of alloxan diabetic rats	161
Angervall L and K Åhrén	Mammary gland development in newborn offspring of alloxan diabetic rats	171
Angervall L and S E Fagerberg	Skin biopsy in diabetes	391
Arnesen Kristen	The cytology of the adrenal cortex in mice with spontaneous adrenocortical lipid depletion	212
Asz H T Å Bergenhoft and O Olsson	Epidermolysis bullosa hereditaria A histopathological study of teeth and oral mucosa in three cases	398
Backgren A L F Johansson A Norden S Rubarth and G Windquist	Spontaneous leucosis in dogs	385
Berglason S and I Dahl	Neoplasms in guinea pigs	154
Bent on		
Bent on		501
Bent on		363
Berg Ole		33
Berge Th and O Grontoft	Cytological diagnosis of pleural mesothelioma	385
Berge Thorbjorn and Nils Söderström	Fine needle cytologic biopsy in diseases of the salivary glands	1
Bergenhoft Å B Olsson and T Arvill	Epidermolysis bullosa hereditaria A histopathological study of teeth and oral mucosa in three cases	398
	Immune effect of lymphoid	156
	Neoplasms in guinea pigs	177
Bjerring F and I Crannet	Quantitative data on the cell content of the femoral bone marrow in the adult female albino rat	272
Bjerring I and N F Borjlin	Eosinophilia in uterus	388

<i>Böttiger Margareta</i> Antibodies after polio vaccination	153
<i>Boire Kjell</i> Affinities between <i>Moraxella</i> spp and a strain of <i>Neisseria catarrhalis</i> as expressed by transformation	528
<i>Bovre Kjell and Asbjørn M Tonjum</i> Non pigmented <i>Serratia marcescens</i> var <i>kielensis</i> as a probable cause of bronchopneumonia	251
<i>Borglin A F and I Bjersing</i> Eosinophilia in uterus	388
<i>Borglin A F and Sten Winblad</i> Studies of antibodies in human listeriosis	
Antibody titres in healthy people and patients of different categories	133
<i>Bucht H B Wehle P Ørsten and I Ø Kallings</i> Treatment of chronic pyelo-nephritis with ampicillin	160
<i>Cederberg A and G Østberg</i> Oxygen intoxication	397
<i>Cederberg Anni Harald Teir and Tapio Rytömaa</i> Observations on effects of ileum resection on granulocyte counts in blood in rat	401
<i>Cedergren Bertil Lars Edebo and John A Rose</i> A simple method to prepare particles in suspension for electron microscopy	336
<i>Christensen Ebbe Ahrensburg</i> Infection and malignant tumours: A comparison between the effect of phagocytes of haemolytic streptococci and Coley mixed toxins on Brown Pearce carcinoma	43
<i>Christensen Ebbe Ahrensburg Knud Skathauge and An Inge Ravn-Jensen</i> Autoclaving of syringes in pressure cookers	521
<i>Dahlgren S</i> Renal cell changes after infusion of low viscosity dextran	399
<i>Danielsson D and G Laurell</i> Rapid detection of small numbers of bacteria in water by means of fluorescent antibodies	159
<i>Dedichen J P Laland and S G Laland</i> The effect of material prepared from ox blood on cold stress in mice	219
<i>Diderholm H and T Wesslen</i> Inhibition of polycyema virus haemagglutination by different tissues of mouse and hamster	153
<i>Edebo Lars John A Rose and Bertil Cedergren</i> A simple method to prepare particles in suspension for electron microscopy	336
<i>Fhrenberg Bengt C G Ahlstrom and Sten Bergman</i> Neoplasms in guinea pigs induced by an agent in Rous chicken sarcoma	177
<i>Fnerback L</i> Histochemical reactions in carcinoids	391
<i>Engstrom V A M Herrlin A Bergstrand and C G Bergstrand</i> Renal changes	
<i>Friksen Jørn and S D Henriksen</i> Immunochemical studies on some serological cross reactions in the <i>Klebsiella</i> group 9 Cross reactions between <i>Klebsiella</i> types 11 21 and a closely related strain	215
<i>Eriksen Knud Rieuerts Ove Jessen Kirsten Rosenfal Viggo Faber and Karl</i> " " related to patho- coccus aureus	81
Staphylococcus aureus strains isolated in Danish hospitals from April 1st	
<i>Eriksen Knud Rieuerts Kirsten Rosenfal Aksel Stenderup and Peder Helms</i> Staphylococcus aureus strains isolated in Danish hospitals from April 1st to December 31st 1960	72
<i>Faber Viggo Karl Høie Knud Rieuerts Friksen Ove Jessen and Kirsten Rosendal</i> Some properties of <i>Staphylococcus aureus</i> possibly related to pathogenicity Part 3 Bacteriological investigations of <i>Staphylococcus aureus</i> strains from 462 cases of bacteraemia	85
<i>Fagerberg S E and I Angervall</i> Skin biopsy in diabetes	391
<i>Fagraeus Astrid O Skold and K Berglund</i> The enhancing effect of lymphoid cells and cell fractions on antibody response	156
<i>Falkmer S and F Knutson</i> Is cobalt concentrated in pancreatic islet tissue?	387
<i>Falkmer S F Knutson and G F Voigt</i> Further studies on the cobalt concentrating ability of pancreatic islet tissue	393
<i>Felbo M J C Nielsen and M Verstrøm</i> On the presence of group specific Gm and Ge substances in urine	264
<i>Fichera G and I Hagerstrand</i> Lymphatic changes in chronic pulmonary congestion	788
<i>Fjellström K F</i> Electrophoretic studies on human complement I: activation of C factors in starch gel	156
<i>Flyger G H Freidenfeldt and S R Orell</i> Intracerebral possibly malignant osteochondrosarcoma in a child	299

<i>Fredensfeldt H S R Orell and G Flyger</i> Intracerebral possibly malignant osteochondrosarcoma in a child	299
<i>Frisk R and H Tunegall</i> Studies on penicillins of the phenoxymethane series	160
<i>Grøntoft H</i> Histo pathological diagnosis of needle biopsies from the prostate gland	385
<i>Grøntoft H and Th Berge</i> Cytological diagnosis of pleural mesothelioma	385
<i>Grøntoft O and S Lundberg</i> A case of bilateral symmetrical calcifications of the brain	388
<i>Grunnet I and F Biering</i> Quantitative data on the cell content of the femoral bone marrow in the adult female albino rat	272
<i>Gydel E I Juhlin and J G Vorden</i> Nocardiosis	396
<i>Hagerstrand I</i> Thalidomid induced malformations	391
<i>Hagerstrand I and H Fichera</i> Lymphatic changes in chronic pulmonary congestion	388
<i>Hagerstrand I and F Linell</i> Sarcoidosis and pollen	388
<i>Hansen Jes and J J Pindborg</i> Studies on odontogenic cyst epithelium 2 Clinical and roentgenologic aspects of odontogenic keratocysts	283
<i>Harboelt F</i> The <i>in vitro</i> demonstration of a cytotoxic factor in Ehrlich's ascites carcinoma	10
<i>Harboelt F</i> The viability of pyknotic Ehrlich ascites carcinoma cells	17
<i>Harboelt F</i> Sex differences in the intraperitoneal growth of Ehrlich's ascites carcinoma	25
<i>Hedestrød B</i> Treponema pallidum immobilization in normal serum	158
<i>Hedström C F and F Lycke</i> An experimental study of oysters as virus carriers	155
<i>Helgeland A H Lohelle and J Jonsen</i> Polyoma virus 1 Cell virus interaction at low temperature	511
<i>Helma Peder Knud Rieverts Friksen Kirsten Rosendal and Aksel Stenderup</i> Staphylococcus aureus strains isolated in Danish hospitals from April 1st to December 31st 1960	72
<i>Henriksen S D and Jorun Eriksen</i> Immunochemical studies on some serological cross reactions in the Klebsiella group 9 Cross reactions between Klebsiella types 11 21 and a closely related strain	245
<i>Hermanson S</i> Inhibition of interferon in double infected cells	156
<i>Herrlin A M 4 Bergstrand C G Bergstrand and V Engstrom</i> Renal changes during Tridione treatment	387
<i>Hjort Tage</i> The thyroglobulin pool in the thyroid gland in patients with and without thyroid auto antibodies	429
<i>Hogman C and J Kullander</i> Fractionation of human blood group antibodies by gel filtration	156
<i>Holm S and F Lycke</i> Studies on vaccinia LS antigen	155
<i>Høne Karl Knud Rieverts Friksen Ole Jensen Kirsten Rosendal and Viggo Faber</i> Some properties of Staphylococcus aureus possibly related to pathogenicity Part 3 Bacteriological investigations of Staphylococcus aureus strains from 462 cases of bacteraemia	85
<i>Huldt Gunnar</i> Experimental toxoplasmosis Parasitaemia in guinea pigs	457
<i>Hultquist G and J Thorell</i> Trials with pancreas transplantation in rats and guinea pigs	386
<i>Jensen J Skafte and H Verstrøm</i> Immuno-electrophoretic analysis of blood stains with special reference to Gc grouping	257
<i>Jensen Ole Kirsten Rosendal Viggo Faber Karl Høne and Knud Rieverts Friksen</i> Some properties of Staphylococcus aureus possibly related to pathogenicity Part 3 Bacteriological investigations of Staphylococcus aureus strains from 462 cases of bacteraemia	85
<i>Johansen I F A Vorden S Rubarth H Winqvist and A Backgren</i> Spontaneous leucosis in dogs	385
<i>Jonsen J A Helgeland and O Lohelle</i> Polyoma virus 1 Cell virus interaction at low temperature	511
<i>Juhlin Ingmar</i> A new fermentation medium for gonorrhoeae HAP medium Influence of differ-	51
<i>Juhlin I J G Vorden</i>	396
<i>Kjellén Bengt and Ole B</i>	33
allergic encephalo	

Kallings L O H Bucht B Wehle and P Örsten Treatment of chronic pyelo nephritis with ampicillin	160
Kallings L O and A Lindberg Role of lysogenicity in O 1 resistant Salmonella bacteria	159
Kauffmann F Zur Differentialdiagnose der Salmonella-Sub Genera I II und III	109
Kauffmann F Supplement to the Kaufmann White scheme (VI)	339
Kauffmann F Zur Serologie des Salmonella Sub Genus II	348
Kauffmann F and A Petersen Zur Serologie der Salmonella O Gruppen 30 42 43 48 und 50	99
Killander J and C Hogman Fractionation of human blood group antibodies by gel filtration	136
Kim Hyun Kyu M Weis Bent on and Mogens Magnusson Tuberculin produc tion 1 Yield of tuberculo-protein from various media	363
Kim Hyun Kyu Mogens Magnusson and M Weis Bent on Tuberculin produc tion 2 Comparison between amount of tuberculin obtained from the culture filtrate and by extraction of tubercle bacilli	501
Klein G A short survey of some main problems in modern experimental tumour virology	152
Knutson F Autoradiography of pituitary cells in adrenalectomized white rats after the injection of adenine <sup>14</sup> C	391
Knutson F and S Falkmer Is cobalt concentrated in pancreatic islet tissue?	387
Knutson F G E Voigt and S Falkmer Further studies on the cobalt concen trating ability of pancreatic islet tissue	393
Lagerlöf B Transplantation of chromosome labelled leukaemia cells	386
Lagerlöf B and P Sunstein Diseases induced by myeloid leukaemia virus	304
Lahelle O J Jonsen and K Hefgeland Polyoma virus 1 Cell virus interaction at low temperature	511
Lahiti Ato Jussi Suukkonen Tapani Vainio and Teijo Allas Double diffusion precipitation and immunoelectrophoresis of F coli B antigens	517
Laland S G J Dedichen and P Laland The effect of material prepared from ox blood on cold stress in mice	219
Laland P S G Laland and J Dedichen The effect of material prepared from ox blood on cold stress in mice	219
Larsson B A case of postangiographic Th deposition and primary squamous cell carcinoma of the liver	389
Larsson B On malignant liver and bile duct tumours in cases exposed to thorotrast and among winegrowers with long standing exposition to arsenic	399
Larsson I F Endocardial myxoma with malignant degeneration	389
Laurell Anna Britta Comparison of the agglutination of FA and FA <sub>11</sub> cells by rheumatoid arthritic sera	157
Laurell G and D Danielsson Rapid detection of small numbers of bacteria in water by means of fluorescent antibodies	159
Leinonen Eero and J Raekallio Adenine triphosphatase activity of rat skin in early wound healing	433
Lindberg A and L O Kallings Role of lysogenicity in O 1 resistant Salmonella bacteria	159
Lindbom C Genesis and intramural spread of a shigellosis epidemic	158
Lindgren Ilmari Dilated renal collecting ducts in a newborn infant as a precursor to medullary sponge kidney Report of a case	293
Sedation and pollen test	388
immunochemical studies in polysacchari lisis 1 Isolation and chemical charac	323
terization	482
Lindbom A and Per Oelting Immunochemical studies in polysacchari	413
2 Antigenic properties	388
bilateral symmetrical calcifications	391
Lundin P The carcinogenic activity of a complex in preparations	389
Lundmark C Carcinoid tumour with metastases in the heart and aortic stenosis	389
Lundmark C Granular atrophy of the brain bilaterating arterial disease	389

<i>Lundmark C</i> A fatal case of Varicella	398
<i>Lycke E and C F Hedstrom</i> An experimental study of oysters as virus carriers	155
<i>Lycke E and S Holm</i> Studies on vaccinia LS antigen	155
<i>Lycke E and Ö Strannegård</i> Studies on antigen antibody systems with reference to mononucleosis infectiosa	156
<i>Magnus Herdis von and Knud Siboni</i> Serodifferentiation between type 1 polio viruses with antisera produced in rabbits using four different immunization schemes	376
<i>Magnusson Mogens M Weis Bentzon and Hyun Kyu Kim</i> Tuberculin production 2 Comparison between amount of tuberculin obtained from the culture filtrate and by extraction of tubercle bacilli	501
<i>Magnusson Mogens Hyun Kyu Kim and M Weis Bentzon</i> Tuberculin production 1 Yield of tuberculoprotein from various media	363
<i>Millett Tore</i> The effect of thalidomide on the growth curve of a riboflavin dependent microbe	355
<i>Munch Bent Staut</i> Complement fixation employed for typing of Coxsackie A viruses isolated from patients	471
<i>Nasiell M</i> The general appearance of the bronchial mucosa in bronchial cancer	385
<i>Nathorst Windahl G</i> Diabetes in pancreatectomized rabbits	386
<i>Verström B M Felbo and J C Nielsen</i> On the presence of group specific Gm- and Cc substances in urine	264
<i>Verström B and J Skafte Jensen</i> Immunoelectrophoretic analysis of blood stains with special reference to Gc grouping	267
<i>Neumüller Carola P ■ Nilsson and H Thörne</i> The antistaphylococcal titre in cattle after subcutaneous and intramuscular treatment respectively, with a combined staphylococcal vaccine	321
<i>Nielsen J C B Verström and M Felbo</i> On the presence of group specific Gm- and Cc substances in urine	264
<i>Nilsson P O H Thörne and Carola Neumüller</i> The antistaphylococcal titre in cattle after subcutaneous and intramuscular treatment respectively, with a combined staphylococcal vaccine	321
<i>Nordbrink Folke</i> Strains of <i>Staphylococcus aureus</i> with increased tolerance to gentian violet	114
<i>Norlen A S Rubarth G Wingvist A Bäckgren and L F Johansson</i> Spontaneous leucosis in dogs	385
<i>Norden J G A Gydell and I Juhlin</i> Nocardiosis	396
<i>Nystrom B</i> Phagocytosis in rheumatoid arthritis	157
<i>Oeding Per and Vortald Losnegard</i> Immunochemical studies on polysaccharides from <i>Staphylococcus epidermidis</i> 1 Isolation and chemical characterization	482
<i>Oeding Per and Vortald Losnegard</i> Immunochemical studies on polysaccharides from <i>Staphylococcus epidermidis</i> 2 Antigenic properties	493
<i>Örsten P I O Kallings H Bucht and B Wehle</i> Treatment of chronic pyelonephritis with ampicillin	160
<i>Östberg G and A Cederberg</i> Oxygen intoxication	397
<i>Ohlson Mariagne</i> Antigenic composition of enteroviruses in immunodiffusion	154
<i>Oker Blom A</i> Interference phenomena with tumour viruses	152
<i>Olling L</i> Perinatal bacterial infections in an autopsy material	389
<i>Olling H</i> Epidermolysis bullosa lethalis some morphological observations	398
<i>Olsson O T Arwilt and A Bergenholz</i> Epidermolysis bullosa hereditaria A histopathological study of teeth and oral mucosa in three cases	398
<i>Orell S R G Flinn and J R</i> Malignant	299
<i>Persson</i>	395
<i>Petersen</i>	30
<i>Philipson I and C</i>	99
<i>Pinborg J J and</i>	154
<i>ical and to</i>	283
<i>Inten Jan and</i>	273
<i>Rous varco</i>	
<i>gressing tur</i>	

<i>Kallings L O H Bucht B Wehle and P Örstén</i> Treatment of chronic pyelonephritis with ampicillin	160
<i>Kallings L O and A Lindberg</i> Role of lysogenicity in O 1 resistant <i>Salmonella</i> bacteria	159
<i>Kauffmann F</i> Zur Differentialdiagnose der <i>Salmonella</i> Sub Genera I II und III	109
<i>Kauffmann F</i> Supplement to the Kauffmann White scheme (VI)	339
<i>Kauffmann F</i> Zur Serologie des <i>Salmonella</i> Sub Genus II	348
<i>Kauffmann F and A Petersen</i> Zur Serologie der <i>Salmonella</i> O Gruppen 30 42 43 48 und 50	99
<i>Killander J and C Hogman</i> Fractionation of human blood group antibodies by gel filtration	156
<i>Kim Hyun Kyu W Weis Bent on and Mogens Magnusson</i> Tuberculin production 1 Yield of tuberculo protein from various media	363
<i>Kim Hyun Kyu Mogens Magnusson and W Weis Bent-on</i> Tuberculin production 2 Comparison between amount of tuberculin obtained from the culture filtrate and by extraction of tubercle bacilli	501
<i>Klein G</i> A short survey of some main problems in modern experimental tumour virology	152
<i>Knutson F</i> Autoradiography of pituitary cells in adrenalectomized white rats after the injection of adenine <sup>14</sup> C	391
<i>Knutson F and S Falkmer</i> Is cobalt concentrated in pancreatic islet tissue?	387
<i>Knutson F G F Voigt and S Falkmer</i> Further studies on the cobalt concentrating ability of pancreatic islet tissue	393
<i>Iagerlöf H</i> Transplantation of chromosome labelled leukaemia cells	386
<i>Iagerlöf B and P Sundelin</i> Diseases induced by myeloid leukaemia virus	394
<i>Ishelle O J Jonsen and K Helgeland</i> Polyoma virus 1 Cell virus interaction at low temperature	511
<i>Ishii Aito Jussi Saukkonen Tapani Vainio and Jyri Atlas</i> Double diffusion of coli B antigens of material prepared from	517
<i>Laland P S G Laland and J Dedichen</i> The effect of material prepared from ox blood on cold stress in mice	219
<i>Larsson B</i> A case of postangiographic Th deposition and primary squamous cell carcinoma of the liver	389
<i>Larsson B</i> On malignant liver and bile duct tumours in cases exposed to thorotrast and among winegrowers with long standing exposition to arsenic	399
<i>Larsson L F</i> Endocardial myxoma with malignant degeneration	359
<i>Laurell Anna Britta</i> Comparison of the agglutination of I A and I A <sub>1</sub> cells by rheumatoid arthritic sera	157
<i>Laurell G and D Danielsson</i> Rapid detection of small numbers of bacteria in water by means of fluorescent antibodies	159
<i>Leinonen Eeva and J Raekallio</i> Adenosine triphosphatase activity of rat skin in early wound healing	471
<i>Lindberg A and L O Kallings</i> Role of lysogenicity in O 1 resistant <i>Salmonella</i> bacteria	159
<i>Lindbom G</i> Genesis and intramural spread of a staphylococcal epidemic	158
<i>Lindgren Ilmari</i> Dilated renal collecting ducts in a newborn infant as a precursor to medullary sponge kidney. Report of a case	297
<i>Lindqvist S and O Grottoft</i> A case of bilateral symmetrical calcifications of the brain	388
<i>Lundin P</i> The carcinogenic action of complex iron preparations	191
<i>Lundmark C</i> Carcinoid tumour with metastases in the heart and aortic stenosis	389
<i>Lundmark C</i> Granular atrophy of the brain obliterating arterial disease	389



<i>Strannegård U</i> and <i>F Lycke</i>	Studies on antigen antibody systems with reference to mononucleosis infectiosa	156
<i>Sundelin P</i> and <i>H Lagerlöf</i>	Diseases induced by myeloid leukaemia virus	394
<i>Sun el H</i> and <i>L Zettergren</i>	Lesions in the liver after surgical operations in the upper part of the abdomen	391
<i>Swart- Malmberg Gunnar</i>	Thermolabile serum factor influencing the neutralization of adenovirus	154
<i>S ögi S</i>	Aseptic meningoencephalitis and myocarditis (probably Coxsackie) in a new born child	388
<i>S ögi S</i>	Isolated toxoplasma myocarditis in a child	399
<i>Ten Harald Tapio Rytömaa</i> and <i>Antti Cederberg</i>	Observations on effects of ileum resection on granulocyte counts in blood in rat	401
<i>Teter Jerry</i>	The mixed germ cell tumours with hormonal activity	306
<i>Thörne H</i> , <i>Carola Neumüller</i> and <i>P O Nilsson</i>	The antistaphylococcal titre in cattle after subcutaneous and intramuscular treatment respectively with a combined staphylococcal vaccine	321
<i>Thorell B</i>	Relationship of virus and cell in experimental leukaemia	152
<i>Thorell B</i>	Localization and determination of respiratory enzymes in the single living cell	387
<i>Thorell J</i>	The diaplacental transfer of insulin antibodies	394
<i>Thorell J</i> and <i>G Hultquist</i>	Trials with pancreas transplantation in rats and guinea pigs	386
<i>Tönjum Asbjörn H</i> and <i>Kjell Bovre</i>	Non pigmented <i>Serratia marcescens</i> var. <i>hielsensis</i> as a probable cause of bronchopneumonia	251
<i>Toonen S L</i> , <i>Saxén</i> and <i>T Väinö</i>	Viral susceptibility and embryonic differentiation 1 The histopathology of mouse kidney rudiments infected with polyoma and vesicular stomatitis viruses <i>in vitro</i>	191
<i>Toivonen S T</i> , <i>Väinö</i> and <i>L Saxén</i>	Viral susceptibility and embryonic differentiation 2 Immunofluorescence studies of viral infection in the developing mouse kidney <i>in vitro</i>	203
<i>Tunevall G</i> and <i>R Frisk</i>	Studies on penicillins of the phenoxymethane series	160
<i>Vainio Tapani</i> , <i>Jyrö Allan</i> , <i>Asto Lahti</i> and <i>Jussi Saukkonen</i>	Double diffusion precipitation and immunoelectrophoresis of F coli B antigens	517
<i>Vainio T</i>	" " " " id embryonic differentiation in the	191
<i>Vainio T</i>	" " " " id embryonic differentiation in the	205
<i>Värfeldt Jakob</i>	Transformation of sympatheticoblastoma into ganglioneuroma With a case report	414
<i>Vogt G E</i> , <i>V Falkner</i> and <i>E Knutson</i>	Further studies on the concentrating ability of pancreatic islet tissue	393
<i>Wehle H</i> , <i>P Östen</i> , <i>L O Hallings</i> and <i>H Bucht</i>	Treatment of chronic pyelonephritis with ampicillin	160
<i>Wesslen T</i> and <i>H Dillerholm</i>	Inhibition of polyoma virus haemagglutination by different tissues of mouse and hamster	153
<i>Wiman L</i>	Sulphydryl chemistry and fluorescence microscopy in exfoliative pulmonary cytology	396
<i>Winblad Sten</i>	Studies of antibodies in human listeriosis 1 Antibodies in cases of bacteriologically verified human listeriosis	123
<i>Winblad Sten</i> and <i>A F Borglin</i>	Studies of antibodies in human listeriosis 2 Antibody titres in healthy people and patients of different categories	133
<i>Winge Helen Kerstin</i>	Sterilization by ethylene oxide without special equipment	160
<i>Winge Hildén Kerstin</i>	Ethylene oxide sterilization without special equipment	225
<i>Wingqvist I</i> , <i>A Backgren</i> , <i>I Johansson</i> , <i>A Norlén</i> and <i>C B A . . .</i>	" " " "	385
<i>Zajicek</i>	" " " "	395
<i>Zamboni</i>	" " " "	385

<i>Raekallio J</i> and <i>Eeva Levenon</i> Adenosine triphosphatase activity of rat skin in early wound healing	451
<i>Rausing A</i> Glioma induced by foreign body	397
<i>Ravn Jonsen Andree Fbbe Ahrensburg Christensen</i> and <i>Knud Skadhauge</i> Autoclaving of syringes in pressure cookers	521
<i>Robertson B</i> Reparative phenomena in hyaline membrane disease	398
<i>Rockert H</i> and <i>L Zettergren</i> Lesions caused by barium contrast media	391
<i>Rockert H</i> and <i>L Zettergren</i> Tissue reaction to barium sulphate contrast medium	445
<i>Rose John K Bertil Cedergren</i> and <i>Lars Edebo</i> A simple method to prepare particles in suspension for electron microscopy	336
<i>Rosendal Kirsten Viggo Faber Karl Høie Knud Riewerts Eriksen</i> and <i>Ole Jessen</i> Some properties of <i>Staphylococcus aureus</i> possibly related to pathogenicity Part 3 Bacteriological investigations of <i>Staphylococcus aureus</i> strains from 42 cases of bacteraemia	85
<i>Rosendal Kirsten Aksel Stenderup Peder Helms</i> and <i>Knud Riewerts Eriksen</i> <i>Staphylococcus aureus</i> strains isolated in Danish hospitals from April 1st to December 31st 1960	72
<i>Rubarth S G Winqvist A Backgren L F Johansson</i> and <i>A Vorden</i> Syon taneous leucosis in dogs	385
<i>Rytomaa Tapio Antti Cederberg</i> and <i>Harald Teir</i> Observations on effects of ileum resection on granulocyte counts in blood in rat	401
<i>Saldeen T</i> Experimental investigation of cancer spread to the liver in rats with liver cirrhosis	395
<i>Santesson L</i> On the classification of ovarian tumours	393
<i>Saukkonen Jussi Tapani Vainio Yrjö Allas</i> and <i>Asto Lahti</i> Double diffusion precipitation and immunoelectrophoresis of <i>E coli</i> B antigens	517
<i>Saxen I S Toivonen</i> and <i>T Vainio</i> Viral susceptibility and embryonic differentiation 2 Immunofluorescence studies of viral infection in the developing mouse kidney <i>in vitro</i>	205
<i>Saxen L T Vainio</i> and <i>S Toivonen</i> Viral susceptibility and embryonic differentiation 1 The histopathology of mouse kidney rudiments infected with polyoma and vesicular stomatitis viruses <i>in vitro</i>	191
<i>Schnürer L B</i> and <i>S Ståltin</i> The vascular supply of tentorial meningiomas	390
<i>Siboni Knud</i> and <i>Herlitz Magnus</i> Serodifferentiation between type 1 polio viruses with antisera produced in rabbits using four different immunization schemes	376
<i>Sjogren O</i> Specific cellular antigens in virus induced tumours	152
<i>Skadhauge Knud Andree Ravn-Jonsen</i> and <i>Fbbe Ahrensburg Christensen</i> Autoclaving of syringes in pressure cookers	521
<i>Sköld O K Birglund</i> and <i>Astrid Fagraeus</i> The enhancing effect of lymphoid cells and cell fractions on antibody response	156
	397
	in diseases
	1
<i>Sourander P</i> Morbus Krahle	391
<i>Ståltin S</i> and <i>L B Schnürer</i> The vascular supply of tentorial meningiomas	390
<i>Stenderup Aksel Peder Helms Knud Riewert Eriksen</i> and <i>Kirsten Rosendal</i> <i>Staphylococcus aureus</i> strains isolated in Danish hospitals from April 1st to December 31st 1960	72
<i>Stenkvist B</i> Growth curves histological appearance and viral titres of Rous sarcomas A comparison between progressive and regressive growing tumours	394
<i>Stenkvist Bjorn</i> and <i>Jan Pontén</i> Growth curves histology and virus titres in Rous sarcomas A comparison between progressively growing and regressing tumours	273
	in Bergman Trials with a rapid method virus determination by microscopic examination in virus inoculated monolayers on slides
	141
	id Stenram Trials with a rapid method virus determination by microscopic examination in virus inoculated monolayers on slides
	141







<i>Heart and aortic stenosis Carcinoid tumour with metastases in the --</i>	by C Lundmark	389
<i>Hormonal activity The mixed germ cell tumours with --</i>	by Jerzy Teter	306
<i>Hyaline membrane disease Reparative phenomena in --</i>	by B Robertson	398
<i>Heum resection on granulocyte counts in blood in rat Observations on effects on --</i>	by Harald Teir Tapio Rytomaa and Antti Cederberg	401
<i>Immunochemical studies on some serological cross reactions in the Klebsiella group 9 Cross reactions between Klebsiella types 11 21 and a closely related strain</i>	By Jorun Friksen and S D Henriksen	245
<i>Immunofluorescence studies of viral infection in the developing mouse kidney in vitro Viral susceptibility and embryonic differentiation 2 --</i>	by T Vainio L Savén and S Toivonen	205
<i>Infection and malignant tumours 4 Comparison between the effect of phage lysates of haemolytic streptococci and Coley mixed toxins on Brown Pearce carcinoma</i>	By Ebbe Ahrensburg Christensen	43
<i>Insulin antibodies The diaplacental transfer of --</i>	by J Thorell	394
<i>Interferon in double infected cells Inhibition of --</i>	by S Hermodsson	156
<i>Iron preparations The carcinogenic action of complex --</i>	by P Lundin	391
		339
		295
		245
<i>Krabbe Morbus --</i>	by P Sourander	391
<i>Leucosis in dogs Spontaneous --</i>	by A Backgren I I Johansson A Nordén S Rubarth and G Wingqvist	385
<i>Leukaemia cells Transplantation of chromosome labelled --</i>	by B Lagerlöf	386
<i>Leukaemia Relationship of virus and cell in experimental</i>	by B Thorell	152
<i>Leukaemia virus Diseases induced by myeloid --</i>	by P Sundelin and B Lagerlöf	394
<i>Listeriosis 1 Antibodies in cases of bacteriologically verified human listeriosis Studies of antibodies in human --</i>	by Sten Winblad	123
<i>Listeriosis 2 Antibody titres in healthy people and patients of different categories Studies of antibodies in human --</i>	by Sten Winblad and A F Borglin	133
<i>Liver A case of postangiographic Th deposition and primary squamous cell carcinoma of the --</i>	by B Larsson	389
<i>Liver after surgical operations in the upper part of the abdomen Lesions in the</i>	by H Sunzel and L Zettergren	391
<i>Liver and bile duct tumours in cases exposed to thorotrast and among wine growers with long standing exposition to arsenic On malignant</i>	by B Larsson	399
<i>Liver cirrhosis Experimental investigation of cancer spread to the liver in rats with --</i>	by T Saldeen	397
<i>Lymphoid cells and cell fractions of antibody response The enhancing effect of --</i>	by M Berglund Astrid Fagraeus and O Sköld	156
<i>Mutagenicity in B 1 resistant Salmonella bacteria Role of</i>	by A Lindberg and L O Kallings	159
<i>Malformations Thalidomid induced</i>	by I Hagerstrand	391
<i>Meningiomas The vascular supply of tentorial</i>	by I B Schnurer and S Stattin	390
		388
		385
		156
		397
		528
		399
<i>Myocarditis in a child Isolated toxic myocarditis</i>	by S Szegő	
<i>Myocarditis (probably Coxsackie) in a new born child Aseptic meningomyelitis and --</i>	by S Szegő	389



Heart and aortic stenosis Carcinoid tumour with metastases in the --	by C Lundmark	389
Hormonal activity The mixed germ cell tumours with --	by Jerzy Teter	306
Hyaline membrane disease Reparative phenomena in --	by B Robertson	398
Ileum resection on granulocyte counts in blood in rat Observations on effects on --	by Harald Teir, Tapio Rytomaa and Antti Cederberg	401
Immunochemical studies on some serological cross reactions in the <i>Klebsiella</i> group 3 Cross reactions between <i>Klebsiella</i> types 11 21 and a closely related strain	By Jorun Friksen and S D Henriksen	245
Immunofluorescence studies of viral infection in the developing mouse kidney in vitro Viral susceptibility and embryonic differentiation 2 --	by T Vainio, L Saxén and S Toivonen	205
Infection and malignant tumours 4 Comparison between the effect of phagolysates of haemolytic streptococci and Coley mixed toxins on Brown Pearce carcinoma	By Ebbe Ahrensburg Christensen	43
Insulin antibodies The placental transfer of --	by J Thorell	394
Interferon in double infected cells Inhibition of --	by S Hermodsson	156
plex --	by P Lundin	391
ducts in a newborn infant as	by F Kauffmann	339
ari Lindgren		295
11 21 and a close		
ological cross reac		245
Krabbe Morbus --	by P Sourander	391
of chromosome labelled --	by A Backgren L I Johansson A Nordén	388
Leukaemia Relationship of virus and cell in experimental --	by B Thorell	152
Leukaemia virus Diseases induced by myeloid --	by P Sundelin and B Lagerlof	394
Listeriosis 1 Antibodies in cases of bacteriologically verified human listeriosis Studies of antibodies in human --	by Sten Winblad	193
Listeriosis 2 Antibody titres in healthy people and patients of different categories Studies of antibodies in human --	by Sten Winblad and V F Borglin	133
of the abdomen Lesions in the		331
Liver and bile duct tumours in cases exposed to thorotrast and among mice growers with long standing exposition to arsenic On malignant --	by B Larsson	399
of cancer spread to the liver in rats		397
body response The enhancing effect		156
is and O Skold		
Lysogenicity in O 1 resistant <i>Salmonella</i> bacteria Role of --	by A Lindhert and L O Hallings	159
Malformations Thalidomid induced	by I Hagerstrand	391
Meningiomas The vascular supply of tentorial	by I B Schnurer and S Mattin	390
Meningoencephalitis and myocarditis (probably Coxsackie) in a newborn child Aseptic --	by S Szogi	398
neural	by Th Berge and O Grontoft	385
itigen antibody systems with reference		156
ke		397
Mononucleosis Splenic rupture in injections --	by J Soderstrom	
<i>Moraxella</i> spp and a strain of <i>Yersinia catarrhalis</i> as expressed by transfor		528
on Affinities between	by Kjell Boyre	399
Myocarditis in a child Isolated toxoplasma --	by S Szogi	
Myocarditis (probably Coxsackie) in a newborn child Aseptic meningoencephalitis and --	by S Szogi	388



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Heart and aortic stenosis Carcinoid tumour with metastases in the --, by C Lundmark	389
Hormonal activity The mixed germ cell tumours with -- by Jerzy Teter	306
Hyaline membrane disease Reparative phenomena in -- by B Robertson	398
Ileum resection on granulocyte counts in blood in rat Observations on effects on -- by Harald Teir, Tapio Rytomaa and Antti Cederberg	401
Immunochemical studies on some serological cross reactions in the klebsiella group 9 Cross reactions between klebsiella typis 11 21 and a closely related strain By Jorun Friksen and S D Henriksen	245
Immunofluorescence studies of viral infection in the developing mouse kidney in vitro Viral susceptibility and embryonic differentiation 2 -- by T Vainio, L Saxon and S Toivonen	205
Infection and malignant tumours 4 Comparison between the effect of phage lysates of haemolytic streptococci and Coley mixed toxins on Brown Pearce carcinoma By Ebbe Ahrensburg Christensen	43
Insulin antibodies The diaplacental transfer of -- by J Thorell	394
Interferon in double infected cells Inhibition of -- by S Hermodsson	156
Iron preparations The carcinogenic action of complex -- by P Lundin Kauff	391
Kidney	339
	295
Klebs	
	245
Krabbe Morbus -- by P Sourander	391
Leucosis in dogs Spontaneous -- by A Backgren L F Johansson A Norden S Rubarth and G Winqvist	395
Leukaemia cells Transplantation of chromosome labelled -- by B Lagerlof	386
Leukaemia Relationship of virus and cell in experimental -- by B Thorell	152
Leukaemia virus Diseases induced by myeloid -- by P Sundelin and B Lagerlof	334
Listeriosis 1 Antibodies in cases of bacteriologically verified human listeriosis Studies of antibodies in human -- by Sten Winblad	123
Listeriosis 2 Antibody titres in healthy people and patients of different categories Studies of antibodies in human -- by Sten Winblad and N T Borglin	133
Liver A case of postangiographic Th deposition and primary squamous cell carcinoma of the -- by H Larsson	389
Liver after surgical operations in the upper part of the abdomen Lesions in the --, by H Svanzel and L Zettergren	391
Liver and bile duct tumours in cases exposed to thorotrast and among wine growers with long standing exposition to arsenic On malignant -- by H Larsson	399
	395
	156
Lysogenicity in D 1 resistant Salmonella bacteria Role of -- by A Lindberg and L O Hallings	150
Malformations Thalidomid induced by I Hagerstrand	391
Meningiomas The vascular supply of tentorial -- by L H Schnurer and S Stattin	390
Meningoencephalitis and myocarditis (probably Coxsackie) in a new born child Aseptic -- by S Szogi	388
	385
	156
	397
Mononucleosis Spleen 11	
Moraxella spp and a strain of Neisseria catarrhalis as expressed by transfor- mation Affinities between -- by Kjell Byre	528
Myocarditis in a child Isolated toxoplasma -- by S Szogi	399
Myocarditis (probably Coxsackie) in a new born child Aseptic meningoenceph- alitis and -- by S Szogi	389

Stomatitis viruses in vitro Viral susceptibility and embryonic differentiation 1 The histopathology of mouse kidney rudiments infected with polyoma and vesicular - , by L. Saxén, T. Vainio and S. Toivonen	191
Stress in mice The effect of material prepared from ox blood on cold - , by J. Dedichen, B. Laland and S. G. Laland	219
Sulphydryl chemistry and fluorescence microscopy in exfoliative pulmonary cytology By L. G. Wiman	306
Sympathicoblastoma into ganglioneuroma With a case report Transformation of - by Jakob Vistfeldt	414
Testes in adult rabbits Effect of diencephalic lesions on - , by L. Ahren	330
Thal domide on the growth curve of a riboflavin dependent microbe The effect of - by Tore Midtvedt	355
Thal domide induced malformation By I. Hagerstrand	331
Thorotrast and among winegrowers with long standing exposition to arsenic On malignant liver and bile duct tumours in cases exposed to - , by B. Larsson	399
Thyroid gland in patients with and without thyroid auto-antibodies The thyroglobulin pool in the - by Tage Hjort	429
Thyroiditis Cytologic diagnosis of subacute and chronic - by P. E. Persson	19a
Toxoplasma myocarditis in a child Isolated - by S. Szogi	393
Toxoplasmosis Parasitaemia in guinea pigs Experimental - by Gunnar Hult	4a7
Transplantation in rats and guinea pigs Trials with pancreas - by G. Hultquist and J. Thorell	386
Treponema pallidum immobilization in normal serum By B. Hedersjedi	158
Tridone treatment Renal changes during - by A. Bergstrand, C. G. Bergstrand, A. Engstrom and K. M. Herrlin	387
Tuberculin production 1 Yield of tuberculo protein from various media By Mogens Magnusson, Hyun Kyu Kim and M. Weis Bentzen	363
	501
	395
Ultracytology A short survey of some main problems in modern experimental - by G. Klein	152
Ultraviruses Interference phenomena with - by A. Oker Blom	151
Tumour with metastases in the heart and aortic stenosis Carcinoid - by C. Lundmark	389
Tumours in cases exposed to thorotrast and among winegrowers with long standing exposition to arsenic On malignant liver and bile duct - by B. Larsson	399
Tumour - - - - - by O. Sjogren	393
Tumour - - - - - by Jerzy Teter	152
Uterine - - - - -	306
Varicella A fatal case of - by C. Lundmark	388
Vaccinia A fatal case of - by C. Lundmark	155
Viral susceptibility and embryonic differentiation 1 The histopathology of mouse kidney rudiments infected with polyoma and vesicular stomatitis viruses in vitro By L. Saxén, T. Vainio and S. Toivonen	398
Viral susceptibility and embryonic differentiation 2 Immunofluorescence studies of viral infection on the developing mouse kidney in vitro By T. Vainio, L. Saxén and S. Toivonen	191
Virology A short survey of some main problems in modern experimental tumour - by G. Klein	20a
Virus and cell in experimental leukaemia Relationship of - by B. Thorell	152
Virus carriers An experimental study of oysters as - by C. F. Hedstrom and J. F. Locke	152
Virus 1 Cell virus interaction of influenza temperature Polyoma - by K. Helge	15a
Janet O. Fabelle and J. Jonsen	511



	191
J Dedichen P Laland and S G Laland	219
<i>Salphdryt chemistry and fluorescence microscopy in exfoliative pulmonary cytology</i> By L-G Wiman	396
<i>Sympathicoblastoma into ganglioneuroma With a case report Transformation of</i> by Jakob Vissfeldt	414
<i>Testes in adult rabbits Effect of diencephalic lesions on--</i> , by L Ahren	390
<i>Thalidomide on the growth curve of a riboflavin dependent microbe The effect of</i> by Tore Midtvedt	355
<i>Thalidomid induced malformation</i> By I Hagerstrand	391
<i>Thorotrast and among winegrowers with long standing exposition to arsenic On malignant liver and bile duct tumours in cases exposed to--</i> by B Larsson	399
<i>Thyroid gland in patients with and without thyroid auto antibodies The thyroglobulin pool in the</i> by Tage Hjort	409
<i>Thyroiditis Cytologic diagnosis of subacute and chronic</i> , by P S Persson	395
<i>Toxoplasma myocarditis in a child Isolated--</i> by S Szol	399
<i>Toxoplasmosis Iarasituemia in guinea pigs Experimental--</i> , by Gunnel Hult	457
<i>Transplantation in rats and guinea pigs Trials with pancreas</i> , by G Hultquist and J Thorell	386
<i>Treponema pallidum immobilization in normal serum</i> By B Hederstedt	158
<i>Tridione treatment Renal changes during--</i> by A Bergstrand C G Bergstrand A Engstrom and H M Herrlin	387
<i>Tuberculin production 1 Yield of tuberculoprotein from various media</i> By Mogens Magnusson Hyun hyu kim and M Weis Bentzon	363
<i>Tuberculin production 2 Comparison between amount of tuberculin obtained from the culture filtrate and by extraction of tubercle bacilli</i> By Hyun hyu kim Mogens Magnusson and M Weis Bentzon	501
<i>Tumour material for biochemical and biological investigations Needle biopsies as a method to obtain human</i> by J Zajicek	395
<i>Tumour virology 1 short survey of some main problems in modern experimental</i> by G Klein	152
<i>Tumour viruses Interference phenomena with</i> by A Oker Blom	152
<i>Tumour with metastases in the heart and aortic stenosis (arcinoid--</i> by C Lundmark	369
<i>Tumours in cases exposed to thorotrast and among winegrowers with long standing exposition to arsenic On malignant liver and bile duct--</i> by B Larsson	399
<i>Tumour</i>	393
<i>Tumour</i>	152
<i>Tumour</i>	306
<i>Tumour</i>	388
<i>Tumour</i>	155
<i>Tumour</i>	398
<i>Viral susceptibility and embryonic differentiation 1 The histopathology of mouse kidney rudiments infected with polyoma and vesicular stomatitis viruses in vitro</i> By L Saxén T Vainio and S Toivonen	191
<i>Viral susceptibility and embryonic differentiation 2 Immunofluorescence studies of viral infection on the developing mouse kidney in vitro</i> By T Vainio L Saxén and S Toivonen	205
<i>Virology 1 short survey of some main problems in modern experimental tumour</i> by G Klein	152
<i>Virus and cell in experimental leukaemia Relationship of</i> by B Thorell	152
<i>Virus carriers An experimental study of oysters as</i> by C E Heistrom and E Lycke	155
<i>Virus 1 Cell virus interaction at low temperature Polyoma--</i> by H Helge laud B Lohelle and J Jonsen	511

<i>Renal changes during Tridione treatment</i> By A Bergstrand C G Bergstrand N Engstrom and K M Herrlin	387
<i>Rheumatoid arthritic sera Comparison of the agglutination of F 4 and F 411<sup>a</sup> cells by --</i> by Anna Britta Laurell	157
<i>Rheumatoid arthritis Phagocytosis in --</i> by B Nyström	157
<i>Riboflavin dependent microbe The effect of thalidomide on the growth curve of a --</i> by Tore Midtvedt	355
<i>Rous chicken sarcoma Neoplasms in guinea pigs induced by an agent in --</i> by C G Ahlstrom Sven Bergman and Bengt Ihrenberg	177
<i>Rous sarcoma in mammals and ducks</i> By C G Ahlstrom	153
<i>Rous sarcomas A comparison between progressive and regressive growing tumours Growth curves histological appearance and viral titres of --</i> by H Stenkvist	394
<i>Rous sarcomas A comparison between progressively growing and regressing tumours Growth curves histology and virus titres in --</i> by Bjorn Stenkvist and Jan Pontén	273
<i>Salivary glands Fine needle cytologic biopsy in diseases of the --</i> by Thorbjorn Berge and Nils Soderstrom	1
<i>Salmonella bacteria Role of lysogenicity in O 1 resistant --</i> by A Lindberg and I O Hallings	159
<i>Salmonella O Gruppen 30 42 43 48 und 50 Zur Serologie der --</i> von I Kauffmann und A Petersen	99
<i>Salmonella Sub Genera I II und III Zur Differentialdiagnose der --</i> von I Kauffmann	109
<i>Salmonella Sub Genus II Zur Serologie des --</i> von F Kauffmann	348
<i>Sarcoidosis and pollen</i> By I Hagerstrand and F Linell	388
<i>Sarcoma in mammals and ducks Rous --</i> by C G Ahlstrom	153
<i>Sarcoma Neoplasms in guinea pigs induced by an agent in Rous --</i> by C G Ahlstrom Sven Bergman and Bengt Ihrenberg	177
<i>Sarcomas A comparison between progressive and regressive growing tumours Growth curves histological appearance and viral titres of Rous --</i> by H Stenkvist	394
<i>Sarcomas A comparison between progressively growing and regressing tumours Growth curves histology and virus titres in Rous --</i> by Bjorn Stenkvist and Jan Pontén	273
<i>Serratia marcescens var. tielensis as a probable cause of bronchopneumonia Von pigmented</i> by Kjell Boyre and Asbjorn M Tonjum	251
<i>Splenic rupture in infectious mononucleosis</i> By J Soderstrom	397
<i>Staphylococcal epidemic The genesis and intramural spread of a --</i> by G Lindbom	158
<i>Staphylococcal vaccine The antistaphylolysin titre in cattle after subcutaneous and intramuscular treatment respectively with a combined --</i> by H Thorne Carola Neumuller and P O Nilsson	321
<i>Staphylococcus aureus possibly related to pathogenicity Part 3 Bacteriological investigations of Staphylococcus aureus strains from 162 cases of bacteraemia Some properties of --</i> by Ove Jessen Kirsten Rosendal Viggo Faber Karl Hove and Knud Riewerts Friksen	85
<i>Staphylococcus aureus strains isolated in Danish hospitals from April 1st to December 31st 1960</i> By Kirsten Rosendal Aksel Stendrup Peder Helms and Knud Riewerts Friksen	72
<i>Staphylococcus aureus with increased tolerance to gentian violet Strains of --</i> by Folke Nordbring	114
<i>Staphylococcus epidermidis 1 Isolation and chemical characterization Immunochemical studies on polysaccharides from --</i> by Norvald Losnegard and Per Oeding	482
<i>Staphylococcus epidermidis 2 Antigenic properties Immunochemical studies</i> by Norvald Losnegard and Per Oeding	493
<i>Staphylococcus epidermidis 3 Without special equipment</i> By Kerstin Winge	160
<i>Sterilization without special equipment Ethylene oxide</i> by Kerstin Winge	227
<i>Sterilization without special equipment</i> by Kerstin Winge	227

<i>Stomatitis viruses in vitro Viral susceptibility and embryonic differentiation</i>	
1 The histopathology of mouse kidney rudiments infected with polyoma and vesicular -- by L. Saxén, T. Vainio and S. Toivonen	191
<i>Stress in mice The effect of material prepared from ox blood on cold --</i> , by J. Dedichen, P. Laland and S. G. Laland	219
<i>Sulphydryl chemistry and fluorescence microscopy in exfoliated pulmonary cytology</i> By L. G. Wiman	396
<i>Sympatric blastoma into ganglioneuroma With a case report Transformation of --</i> by Jakob Viskeldt	414
<i>Testes in adult rabbits Effect of diencephalic lesions on --</i> , by C. Ahren	390
<i>Thalidomide on the growth curve of a riboflavin dependent microbe The effect of --</i> by Tore Midtvedt	305
<i>Thalidomid induced malformation</i> By I. Hagerstrand	391
<i>Thorotrast and among winegrowers with long standing exposition to arsenic On malignant liver and bile duct tumours in cases exposed to --</i> , by B. Larsson	399
<i>Thyroid gland in patients with and without thyroid auto antibodies The thyroglobulin pool in the --</i> by Tage Hjort	429
<i>Thyroiditis Cytologic diagnosis of subacute and chronic --</i> by P. S. Persson	390
<i>Toxoplasma myocarditis in a child Is fatal --</i> by S. Szogö	399
<i>Toxoplasmosis Parasitaemia in guinea pigs Experimental --</i> by Gunnel Hult	407
<i>Transplantation in rats and guinea pigs Trials with pancreas --</i> by G. Hultquist and J. Thorell	386
<i>Treponema pallidum immobilization in normal serum</i> By B. Hederstedt	108
<i>Tridione treatment Renal changes during --</i> by A. Bergstrand, C. G. Bergstrand, E. Engström and K. W. Herrlin	387
<i>Tuberculin production 1 Yield of tuberculoprotein from various media</i> By Mogens Magnusson, Hyun hyu kim and M. Weis Bentzen	363
<i>Tuberculin production 2 Comparison between amount of tuberculin obtained from the culture filtrate and by extraction of tubercle bacilli</i> By Hyun hyu kim, Mogens Magnusson and M. Weis Bentzen	501
<i>Tumour material for biochemical and biological investigations Needle biopsies as a method to obtain human --</i> by J. Zajicek	390
<i>Tumour virology A short survey of some main problems in modern experimental --</i> by G. Klein	102
<i>Tumour viruses Interference phenomena with --</i> by N. Ober Blom	152
<i>Tumour with metastases in the heart and aortic stenosis Carcinoid --</i> by C. Lundmark	389
<i>Tumours in cases exposed to thorotrast and among winegrowers with long standing exposition to arsenic On malignant liver and bile duct --</i> by B. Larsson	399
<i>Tumours On the classification of ovarian --</i> by I. Santesson	393
<i>Tumours Specific cellular antigens in virus induced --</i> by O. Sjogren	132
<i>Tumours with hormonal activity The mixed germ cell --</i> by Jerry Teter	306
<i>Uterus Eosinophilia in --</i> by I. Bjersing and N. L. Borghu	388
<i>Vaccinia LS antigen Studies on --</i> by S. Holm and F. Ljéke	155
<i>Vaccinia A fatal case of --</i> by C. Lundmark	398
<i>Viral ... and ...</i>	191
<i>Virus ... mouse kidney in vitro</i> By T. Vainio, I. Saxén and S. Toivonen	200
<i>Virus ... short survey of some main problems in modern experimental ...</i> by G. Klein	102
<i>Virus ... in experimental leukaemia Relationship of --</i> by H. Thorell	152
<i>Virus carriers An experimental study of oysters as --</i> by C. E. Hedström and I. Ljéke	155
<i>Virus ... Cell virus interaction at low temperature Polyoma --</i> by K. Helge	155
<i>Virus ... I. Labelle and J. Jonsen</i>	

<i>Renal changes during Tridione treatment</i> By A Bergstrand C G Bergstrand N Engstrom and K M Herrlin	387
<i>Rheumatoid arthritic sera Comparison of the agglutination of FA and FA<sub>11</sub> cells by --</i> by Anna Britta Laurell	157
<i>Rheumatoid arthritis Phagocytosis in --</i> by E Nyström	157
<i>Riboflavin dependent microbe The effect of thalidomide on the growth curve of a --</i> by Tore Midtvedt	355
<i>Rous chicken sarcoma Neoplasms in guinea pigs induced by an agent in --</i> by C G Ahlström Sven Bergman and Bengt Ehrenberg	177
<i>Rous sarcoma in mammals and ducks</i> By C G Ahlström	153
<i>Rous sarcomas A comparison between progressive and regressive growing tumours Growth curves histological appearance and viral titres of --</i> by B Stenkvist	394
<i>Rous sarcomas A comparison between progressively growing and regressing tumours Growth curves histology and virus titres in --</i> by Björn Stenkvist and Jan Pontén	273
<i>Salivary glands Fine needle cytologic biopsy in diseases of the --</i> by Thorbjörn Berge and Nils Söderström	1
<i>Salmonella bacteria Role of lysogenicity in O 1 resistant --</i> by A Lindberg and L O Kallings	159
<i>Salmonella O Gruppen 30 42 43 48 und 50 Zur Serologie der --</i> von F Kauffmann und A Petersen	99
<i>Salmonella Sub Genera I II und III Zur Differentialdiagnose der --</i> von F Kauffmann	109
<i>Salmonella sub Genus II Zur Serologie des --</i> von F Kauffmann	348
<i>Sarcoidosis and pollen</i> By I Hagerstrand and F Linell	388
<i>Sarcoma in mammals and ducks Rous</i> by C G Ahlström	153
<i>Sarcoma Neoplasms in guinea pigs induced by an agent in Rous</i> by C G Ahlström Sven Bergman and Bengt Ehrenberg	177
<i>Sarcomas A comparison between progressive and regressive growing tumours Growth curves histological appearance and viral titres of Rous</i> by B Stenkvist	394
<i>Sarcomas A comparison between progressively growing and regressing tumours Growth curves histology and virus titres in Rous</i> by Björn Stenkvist and Jan Pontén	273
<i>Serratia marcescens var. Lielensis as a probable cause of bronchopneumonia Non pigmented</i> by Kjell Boyre and Asbjörn M Tonjum	251
<i>Splenic rupture in infectious mononucleosis</i> By J Söderström	397
<i>Staphylococcal epidemic The genesis and intramural spread of a --</i> by G Lindbom	158
<i>Staphylococcal vaccine The antistaphylolysin titre in cattle after subcutaneous and intramuscular treatment respectively with a combined --</i> by H Thorne Carola Neumüller and P O Nilsson	321
<i>Staphylococcus aureus possibly related to pathogenicity Part 3 Bacteriological investigations of Staphylococcus aureus strains from 462 cases of bacteraemia Some properties of --</i> by Ove Jessen Kirsten Rosendal Viggo Faber Karl Hove and Knud Riewerts Friksen	85
<i>Staphylococcus aureus strains isolated in Danish hospitals from April 1st to December 31st 1960</i> By Kirsten Rosendal Aksel Stenderup Peder Helms and Knud Riewerts Eriksen	72
<i>Staphylococcus aureus with increased tolerance to gentian violet Strains of --</i> by Folke Nordbrink	114
<i>Staphylococcus epidermidis 1 Isolation and chemical characterization Immunochemical studies on polysaccharides from --</i> by Norvald Losnegård and Per Oeding	481
<i>Staphylococcus epidermidis 2 Antigenic properties Immunochemical studies on polysaccharides from --</i> by Norvald Losnegård and Per Oeding	493
<i>Sterilization by ethylene oxide without special equipment</i> By Kerstin Winkö Hedén	160
<i>Sterilization without special equipment Ethylene oxide</i> by Kerstin Winkö Hedén	255





<i>Virus determination by microscopic examination of the individual cells in virus inoculated monolayers on slides Trials with a rapid method for quantitative and qualitative--</i>	by Sven Bergman Ingrid Stenram and Unni Stenram	141
<i>Virus Diseases induced by myeloid leukaemia--</i>	by P Sundelin and B Lagerlöf	394
<i>Virus haemagglutination by different tissues of mouse and hamster Inhibition of polyoma--</i>	by H Diderholm and T Wesslén	153
<i>Virus induced tumours Specific cellular antigens in --</i>	by O Sjögren	152
<i>Virus infection in mice Electron microscopical investigation on the early stages of polyoma--</i>	by L Zamboni	387
<i>Virus Thermolabile serum factor influencing the neutralization of adeno--</i>	by Gunvor Svartz Malmberg	154
<i>Virus type I Counter current distribution of polyo--</i>	by S Bengtsson and I Philipson	154
<i>Viruses in immunodiffusion Antigenic composition of entero--</i>	by Marianne Ohlson	154
<i>Viruses Interference phenomena with tumour--</i>	by N Oker Blom	152
<i>Viruses isolated from patients Complement fixation employed for typing of Coxsackie A--</i>	by Bente Stautz Much	471
<i>Voges Proskauer test Technique of--</i>	by V Ijutov	325
<i>Wound healing Adenosine triphosphatase activity of rat skin in early--</i>	by J Ruckallio and Eeva Levenon	451

# SUPPLEMENTA

Supplementum 162	<i>Saldeen Tom</i> Experimental studies on spread of Rous sarcoma in rats Pp 87, 1963
Supplementum 163	<i>Stormby V G</i> Effect of sodium salicylate on tumour transplantation in rats Pp 76 1963
Supplementum 164	<i>Fortelius Pir</i> Enzyme activity in cultured cells under various influences A cytochemical approach Pp 94 1963
Supplementum 165	<i>Iversen Olaf Hilmar and Rolf Bjerknes</i> Kinetics of epidermal reaction to carcinogens A cybernetic model analysis Pp 74 1963

